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ANTIHYALURONIDASE ANTAGONISTIC TO PNEUMOCOCCUS HYALURONIDASE IN THE SERUM OF NORMAL HUMAN BEINGS AND PATIENTS WITH PNEUMOCOCCIC PNEUMONIA: RISE OF TITER IN BACTEREMIC PNEUMOCOCCIC PNEUMONIA

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THE problem of rapidly fatal pneumococcic pneumonia at the Cincinnati General Hospital has prompted the present inquiry into the pathogenesis of this disease. Approximately one-fourth of the fatalities due to pneumococcic pneumonia at this hospital in the last three years occurred within twenty-four hours of the patient's admission.[1, 2] Between July 1, 1945, and July 1, 1947, there were 426 patients with pneumococcic pneumonia admitted, including those with purulent complication on admission; the mortality has been 15 per cent.[2]

In 1931, from a study of human autopsy specimens, Loeschcke[3] concluded that actively advancing pneumococcic pneumonia spread within a lobe of lung by progression of a peripheral zone of edema fluid containing pneumococci from alveolus to alveolus through the pores of Kohn. This was corroborated experimentally by Robertson, Coggeshall, and Terrell,[4] Gunn and Nungester,[5] and Wood.[6] These investigators found that spreading experimental pneumococcic pneumonia was marked by a peripheral zone of alveoli containing fluid and pneumococci which preceded definite change in the appearance of the alveolar cells or the migration of erythrocytes and polymorphonuclear leucocytes into the alveoli. Robertson and associates[4] also described regurgitation of this infected alveolar fluid through communicating bronchioles into previously uninvolved alveoli of the same lobe of lung. Robertson and Hamburger[7] described the interlobar spread of experimental pneumococcic pneumonia by the intrabronchial flow of infected fluid exudate.

Filtrates of virulent cultures of pneumococcus contain spreading factor (diffusion factor) which produces edema when injected into the flank skin of the rabbit.[8] This edema producing effect of spreading factor was demonstrated to be enzymic when Chain and Duthie[14] established the identity of spreading factor and hyaluronidase in 1939. In a survey of in vitro hyaluronidase production by eighty-one strains of pneumococcus isolated from successive cases of pneumonia, Humphrey found no correlation between the amount of hyaluronidase produced and the clinical severity of pneumonia.[17] However the in vitro production of hyaluronidase by pneumococci, which varies according to the availability of hyaluronic acid in the medium and the duration of incubation of the

This work was done in the Departments of Internal Medicine and Biochemistry of the College of Medicine of the University of Cincinnati, and the Cincinnati General Hospital.

Technical assistance was given by Barbara Taylor, B.A., Barbara Moulton, B.S., and Frances E. Moses, B.S.

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culture, may not truly reflect the elaboration of hyaluronidase during pneumococcic infection. Therefore the present investigation was undertaken to study in vivo elaboration of hyaluronidase during pneumococcic pneumonia in man.

Spreading factor was first described by Duran-Reynals in 1928[9] when the lesion of vaccinial infection of the rabbit was enlarged by aqueous extracts of rabbit, guinea pig, and rat testicle. The spreading effect was characterized by a zone of edema and was ascribed to an increase of tissue permeability by McClean[10] and by Hoffman and Duran-Reynals.[11] Duran-Reynals found this spreading factor in filtrates of only invasive strains of staphylococci and streptococci and not in noninvasive strains.[12]

Hyaluronidase is the enzyme which hydrolyzes the viscous mucopolysaccharide called hyaluronic acid.[13] Hyaluronic acid, the substrate, has been extracted from mammalian vitreous humor, synovial fluid, umbilical cord,[13] and skin.[14] There is indirect evidence of the presence of hyaluronic acid in lung tissue.[17] Hyaluronic acid is soluble in water and will not dialyze through a porous cellophane membrane. The exact chemical composition of hyaluronic acid is undetermined, but it is known to contain equimolar amounts of hexosamine, acetyl, and glucuronic acid.[15] Hyaluronic acid, the substrate, is not antigenic,[16] in contradistinction to hyaluronidase, the enzyme, which is antigenic.[20] The studies of Bensley[18] suggest that hyaluronic acid is one of the components of the intercellular ground substance of loose connective tissue. Accepting the probability of this premise, Duran-Reynals has proposed the hypothesis that infection by invasive bacteria is induced by hydrolysis of the mucoid ground substance of connective tissue.[19]

Hyaluronidases are antigenic, as demonstrated by McClean in 1943.[20] Therefore it was proposed to estimate the amount of pneumococcus hyaluronidase elaborated in selected cases of pneumococcic pneumonia by titration of the corresponding antihyaluronidase which was elaborated in the serum of each patient. A series of sera was drawn on each patient tested and rises of antihyaluronidase titer in a given patient's series of sera were taken to indicate elaboration of hyaluronidase during that patient's infection.

Three groups of patients, representing three rather definite degrees of severity of primary pneumococcic pneumonia, were selected for these tests. Qualifications of the patients selected for the respective groups were as follows: Negative blood culture and no purulent complication, pneumococcus bacteremia and no purulent complication, and pneumonoccus bacteremia with purulent complication. All of these patients were studied while they were under treatment for pneumonia at the Cincinnati General Hospital. All patients included here had blood culture taken on admission to the hospital, and initial serum was drawn within three days of admission.

A preliminary report of this work was published in abstract form in 1946,[21*] and a later report was published in abstract form in 1947.[22*]

*These reports were read at the 1945 and the 1947 Meetings of the Central Society for Clinical Research.

METHODS

Serial dilutions of the serum to be tested were incubated with a constant amount of hyaluronidase, and then these incubates were tested for residual hyaluronidase activity by Byers' modification[23] of McClean's mucoprotein clot prevention (M.C.P.) test.[20] The highest dilution of the serum which inactivated a constant amount of hyaluronidase was taken as the antihyaluronidase titer of that serum.

The hyaluronidases used in these tests were Seitz filtered seventy-two hour cultures of mouse virulent Types 1, 2, and 7 pneumococci in fluid medium which contained 0.75 per cent potassium hyaluronate and no glucose. The potassium hyaluronate used was prepared by the method of Byers, Tytell, and Logan.[24] The three hyaluronidases used in the preliminary tests[21] were prepared by Gibert in the semisynthetic medium which she developed for pneumococci.[25] The three hyaluronidases used in subsequent tests were prepared in beef heart infusion broth (Difco). By the mucoprotein clot prevention test five of these hyaluronidases were active in the dilution of at least 1:16,000, and the sixth was active in the dilution of 1:1,200.

The constant amount of hyaluronidase used throughout these tests was 0.25 c.c. of the unrefined hyaluronidase which had been diluted so that a 1:8 or a 1:16 dilution prevented the mucoprotein clot, regardless of the original potency of the preparation used. This dilution of each hyaluronidase was freshly prepared each day and was used to supply all tests done that day. All dilutions of hyaluronidase and of serum to be tested were made in 1 per cent proteose peptone in physiologic saline. This solution minimized a partial loss of potency which occurred when the hyaluronidases were diluted in distilled water or physiologic saline.

All of the sera in one patient's series of sera were tested at one time with one hyaluronidase preparation. Five to seven normal sera and the serum of Patient A. T.* as the control were tested at one time with the Type 2 pneumococcus hyaluronidase. Twofold serial dilution, beginning with 1:2, was made for each serum tested. Usually ten dilutions were made for each serum, but more were added as needed to obtain an end point. Serum dilutions were made in the volume of 0.5 c.c. in tubes 100 by 13 millimeters. The constant amount of hyaluronidase in the volume of 0.25 c.c. was added to each serum dilution and to a control tube containing 0.5 c.c. of proteose peptone saline with no serum. The tubes were mixed by shaking the tube rack; then they were incubated at room temperature (25° C.) for twenty minutes. The final dilution of serum in the first tube was 1:3, and the final volume in each tube was 0.75 cubic centimeter.

Residual hyaluronidase activity which remained after incubation of the serum dilution and hyaluronidase mixtures was determined as follows:

(1) To each tube was added 1.0 c.c. of substrate. This substrate contained equal volumes of three components: dialyzed tryptic digest of umbilical cord

*This patient was six months convalescent from Type 2 pneumococcus tricuspid endocarditis with repeated septic pulmonary emboli which were complicated by Type 2 pneumococcus empyema.

which contained approximately 0.20 per cent hyaluronic acid,[24] normal horse serum which exhibited neither hyaluronidase nor antihyaluronidase activity, and distilled water.

(2) The tubes were mixed by shaking the tube rack. They were incubated in a water bath at 37° C. for thirty minutes, and then cooled in an ice water bath for five minutes.

(3) To each tube was added 1.0 c.c. of 5 per cent acetic acid, and each tube was shaken individually and read for formation of the mucoprotein clot. The highest dilution of a given serum which exhibited either heavy or thready clot was taken as the antihyaluronidase titer of that serum. Results were expressed in terms of dilution of serum prior to addition of the substrate. Activity of the constant amount of hyaluronidase was demonstrated in the control tube by prevention of the clot.

The mucoprotein clot prevention had a standard error of about 22 per cent as estimated by McClean in his own use of the test.[20] The determination of antihyaluronidase, as done here, permitted a twofold chance variation of titer of a given serum, but fourfold variation did not occur. For example, antihyaluronidase determinations were repeated eight times on the serum of Patient A. T. in its use as a control serum, with titers of 1:384 six times and titers of 1:192 two times.

TABLE I. ANTIHYALURONIDASE ANTAGONISTIC TO PNEUMOCOCCUS HYALURONIDASE (FROM TYPE 2 PNEUMOCOCCUS); SERUM TITERS OF NORMAL HUMAN BEINGS COMPARED WITH THE SERUM TITERS OF CONVALESCENT PATIENT A. T.*

NO.	TITER†		NORMAL: A. T.	NO.	TITER†		NORMAL: A. T.
	NORMAL SUBJECT	PATIENT A. T.			NORMAL SUBJECT	PATIENT A. T.	
1	1:192	1:192	1	26	1:48	1:384	1:8
2	1:192	1:192	1	27	1:48	1:384	1:8
3	1:192	1:384	1:2	28	1:48	1:384	1:8
4	1:192	1:384	1:2	29	1:48	1:384	1:8
5	1:96	1:192	1:2	30	1:48	1:384	1:8
6	1:96	1:192	1:2	31	1:24	1:192	1:8
7	1:96	1:384	1:4	32	1:24	1:192	1:8
8	1:96	1:384	1:4	33	1:24	1:384	1:16
9	1:96	1:384	1:4	34	1:24	1:384	1:16
10	1:96	1:384	1:4	35	1:24	1:384	1:16
11	1:96	1:384	1:4	36	1:24	1:384	1:16
12	1:96	1:384	1:4	37	1:24	1:384	1:16
13	1:96	1:384	1:4	38	1:24	1:384	1:16
14	1:48	1:192	1:4	39	1:12	1:196	1:16
15	1:48	1:192	1:4	40	1:12	1:384	1:32
16	1:48	1:192	1:4	41	1:12	1:384	1:32
17	1:48	1:384	1:8	42	1:12	1:384	1:32
18	1:48	1:384	1:8	43	1:12	1:384	1:32
19	1:48	1:384	1:8	44	1:12	1:384	1:32
20	1:48	1:384	1:8	45	1:12	1:384	1:32
21	1:48	1:384	1:8	46	1:12	1:384	1:32
22	1:48	1:384	1:8	47	1:12	1:384	1:32
23	1:48	1:384	1:8	48	1:6	1:192	1:32
24	1:48	1:384	1:8	49	1:6	1:384	1:64
25	1:48	1:384	1:8	50	1:6	1:384	1:64

*This patient was six months convalescent from Type 2 pneumococcus tricuspid endocarditis and multiple septic pulmonary emboli with Type 2 pneumococcus empyema.

†The highest dilution of the serum which inactivated the constant amount of hyaluronidase used in the test.

RESULTS

Normal Human Serum.—Antihyaluronidase antagonistic to Type 2 pneumococcus hyaluronidase was present in the serum of two normal human beings in the same titer as that of Patient A. T.; four were ½ the titer of Patient A. T., ten were ¼, sixteen were ⅛, seven were 1/16, nine were 1/32, and two were 1/64 (Table I).

Serum of Pneumococcic Pneumonia Patients.—Serial sera of twenty-six patients with pneumonia were tested: seven patients had negative blood culture and no purulent complication; eleven patients had pneumococcus bacteremia and no purulent complication; eight patients had pneumococcus bacteremia with purulent complications.

TABLE II. ANTIHYALURONIDASE IN PNEUMOCOCCIC PNEUMONIA; BLOOD CULTURE NEGATIVE AND NO PURULENT COMPLICATIONS; TABULATION OF SERUM ANTIHYALURONIDASE TITERS PLOTTED ON THE GRAPHS IN FIG. 1.

PN. TYPE	DAYS ILL BEFORE ADMISSION	DAYS FROM ADMISSION TO FIRST SERUM	DAYS FROM FIRST SERUM TO HIGHEST TITER	TYPE OF PN. HYALURONIDASE ANTAGONIZED	TITER OF FIRST SERUM	TITER OF HIGHEST TITER
3	7	0	18	1	12	12
			18	2	12	12
18	10	1	21	1	24	48
			21	2	24	48
1	3	0	32	7	48	96
7	1	0	19	2	384	384
			19	7	192	192
			5	1	192	384
20	Unknown	3	14*	2	96	48*
			5	7	96	192
7	4	0	8*	1	24	6*
			8*	2	24	12*
6†	2	1	10*	1	384	192*
			10	2	192	192
			10*	7	96	48*

*Antihyaluronidase titers decreased.
†This patient died on the fifteenth hospital day of left ventricular cardiac failure.

Serum of Patients With Negative Blood Culture and no Purulent Complication: Antihyaluronidase responses obtained in the seven patients in this group are presented in Table II and Fig. 1, and the sequence of cases in Table II corresponds with the sequence of graphs in Fig. 1. The sera of two patients were titrated against Type 1, Type 2, and Type 7 pneumococcus hyaluronidases; sera of four patients were titrated against two of these hyaluronidases, and sera of one patient were titrated against only one hyaluronidase. Changes of antihyaluronidase titer found in these fifteen titrations of serial sera were one-fourth fold once, one-half fold four times, unchanged five times, and two-fold five times. The abscissa of each graph represents the time in days subsequent to hospital admission at which serum samples were drawn from the patient. The ordinate represents the antihyaluronidase titers of the sera tested, expressed as the reciprocal of the highest dilutions of sera which inactivated the constant amount of hyaluronidase. Each curve represents the antihyal-

uronidase response as titrated against one of the hyaluronidases. From the graphs in Fig. 1 it can be seen that none of these patients exhibited rise of serum antihyaluronidase titer which exceeded twofold, representing no rise within the accuracy of the test.

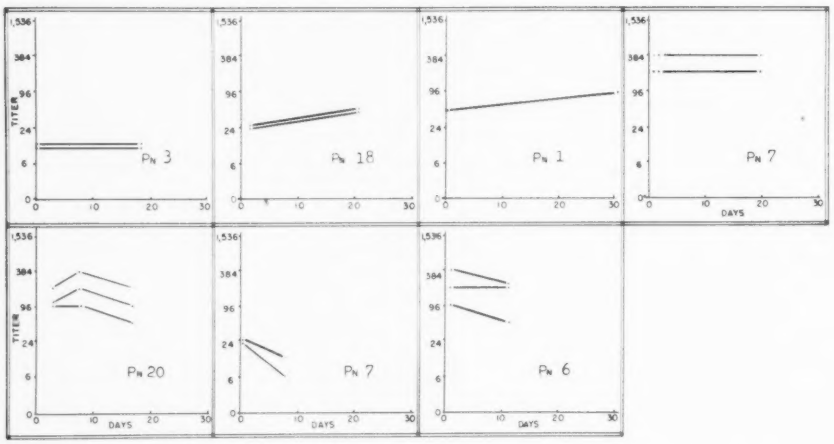

Fig. 1.—Antihyaluronidase in pneumococcic pneumonia; blood culture negative.

The clinical data pertaining to these seven patients are shown in Table III. Their average age was 45.7 years. An average of 1.7 lobes was involved by pneumonia,* and the average hospital stay was 16.4 days. The only death occurred in a 72-year-old woman who died of left ventricular cardiac failure on her fifteenth hospital day, four days after roentgenogram had shown marked resolution of the pneumonia.

TABLE III. ANTIHYALURONIDASE IN PNEUMOCOCCIC PNEUMONIA; BLOOD CULTURE NEGATIVE AND NO PURULENT COMPLICATIONS; CLINICAL DATA

PN. TYPE	AGE OF PATIENT (YR.)	LOBES OF LUNG INVOLVED	TREATMENT*			COMPLICATIONS	DAYS IN HOSPITAL
			SULFA.	PENICILLIN	SERUM		
3	74	LU,RL	+	0	0	0	29
18	29	RU	+	0	0	0	17
1	33	RL	+	0	0	0	11
7	32	LL, RL	+	0	+	0	13
20	54	LL, RU, RL	+	+	0	0	23
7	26	LL	+	0	0	0	7
6†	72	LL, RL	+	+	0	0	15
Average	45.7	1.71					16.4

*Sulpha., Ordinary doses of sulfamerazine[1] or sulfadiazine; Penicillin, 20,000 to 25,000 units of amorphous penicillin every three hours intramuscularly; Serum, type-specific therapeutic antipneumococcic rabbit serum (Lederle).

†This patient died on the fifteenth hospital day of left ventricular cardiac failure.

Serum of Patients With Pneumococcus Bacteremia and no Purulent Complication: Antihyaluronidase responses obtained in the eleven patients in this group are presented in Table IV and Fig. 2, and the sequence of cases in Table

*No distinction was made in this or the other groups of patients as to whether distribution of pneumonia was lobar or bronchial. The type of infecting pneumococcus was identified in all cases, and the distribution of the pneumonia was identified by roentgenogram in all cases.

IV corresponds with the sequence of graphs in Fig. 2. The sera were titrated against Type 1, Type 2, and Type 7 pneumococcus hyaluronidases. Rises of antihyaluronidase titer found in these thirty-three titrations of serial sera were: no change once, twofold seven times, fourfold six times, eightfold eleven times, and sixteen fold eight times. From the curves in Fig. 2 it can be seen that the first eight patients exhibited rises of titer which were fourfold or greater, and that six of these eight patients exhibited rises which were eightfold or greater. These increases of antihyaluronidase titer were all apparent during the patients' first week of hospitalization and were not specific for pneumococcus type. In four patients who were observed for more than thirty days the highest rise of serum antihyaluronidase titer occurred thirteen to thirty days after pneumococcus bacteremia. The last three patients in this group exhibited rises of titer which were twofold or less, representing no rise within the accuracy of the test.

TABLE IV. ANTIHYALURONIDASE IN PNEUMOCOCCIC PNEUMONIA; PNEUMOCOCCUS BACTEREMIA WITHOUT PURULENT COMPLICATIONS; TABULATION OF SERUM ANTIHYALURONIDASE TITERS PLOTTED ON GRAPHS IN FIG. 2

PN. TYPE*	DAYS ILL BEFORE ADMISSION	DAYS FROM ADMISSION TO FIRST SERUM	DAYS FROM FIRST SERUM TO HIGHEST TITER	TYPE OF PN. HYALURONIDASE ANTAGONIZED	TITER OF FIRST SERUM	TITER OF HIGHEST TITER
7	5	1	12	1	24	96
			12	2	12	96
			12	7	24	192
25	4	2	21	1	48	768
			21	2	48	384
			21	7	48	384
3	Unknown	1	13	1	96	1,536
			13	2	192	768
			13	7	96	768
7	5	1	14	1	24	384
			28	2	12	192
			28	7	24	192
12	1	1	17	1	96	1,536
			30	2	96	768
			30	7	48	384
14	Unknown	3	15	1	96	768
			29	2	48	768
			29	7	48	768
7	3	2	18	1	96	768
			21	2	96	768
			18	7	24	384
1	3	2	21	1	48	192
			21	2	96	384
			21	7	48	192
1	0	1	6	1	96	192
			6	2	96	192
			6	7	48	192
25	Unknown	1	18	1	96	192
			18	2	48	96
			18	7	24	48
8	5	2	14	1	96	192
			14	2	96	96
			14	7	96	192

*All of these patients recovered.

The clinical data pertaining to these eleven patients are shown in Table V. The average age was 45.2 years. An average of 1.6 lobes was involved by pneumonia, and the average hospital stay was 29.0 days. All of these eleven patients recovered.

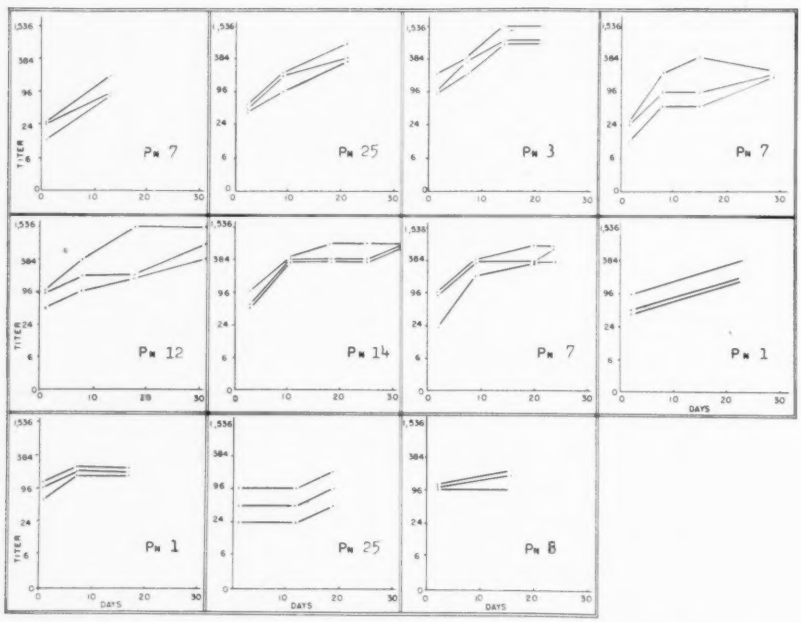

Fig. 2.—Antihyaluronidase in pneumococcic pneumonia; pneumococcus bacteremia without purulent complications.

TABLE V. ANTIHYALURONIDASE IN PNEUMOCOCCIC PNEUMONIA; PNEUMOCOCCUS BACTEREMIA WITHOUT PURULENT COMPLICATIONS; CLINICAL DATA

PN. TYPE*	AGE OF PATIENT (YR.)	LOBES OF LUNG INVOLVED	TREATMENT†			COMPLICATIONS	DAYS IN HOSPITAL
			SULFA.	PENICILLIN	SERUM		
7	37	RU, RM, RL	+	+	+	Cerebral thrombosis‡	40
25	42	RL	+	+	0	Azotemia	37
3	45	LL, RU	+	0	0	Azotemia	24
7	58	RU, RM	+	+	0	Pleural effusion	65
12	35	RU	+	+	0	0	10
14	58	RM, RL	+	+	0	Pulmonary infarction‡	46
7	44	RL	+	+	0	Delayed resolution	28
1	49	RM, RL	+	0	0	0	9
1	18	LL	+	+	0	0	19
25	65	LU, LL	+	+	0	Azotemia	33
8	47	RU	+	0	0	0	9
Average	45.2	1.63					29.0

*All of these patients recovered.

†Sulfa., Ordinary doses of sulfamerazine[1] or sulfadiazine; penicillin, 20,000 to 25,000 units of amorphous penicillin every three hours intramuscularly; serum, type-specific therapeutic antipneumococcic rabbit serum (Lederle).

‡These complications occurred during convalescence.

Serum of Patients With Pneumococcus Bacteremia With Purulent Complications: Antihyaluronidase responses obtained in the eight patients in this group are presented in Table VI and Fig. 3, and the sequence of cases in Table VI corresponds with the sequence of graphs in Fig. 3. The sera were titrated against Type 1, Type 2, and Type 7 pneumococcus hyaluronidases. Rises of antihyaluronidase titer found in these twenty-four titrations of serial sera were fourfold six times, eightfold five times, sixteen fold three times, thirty-two fold

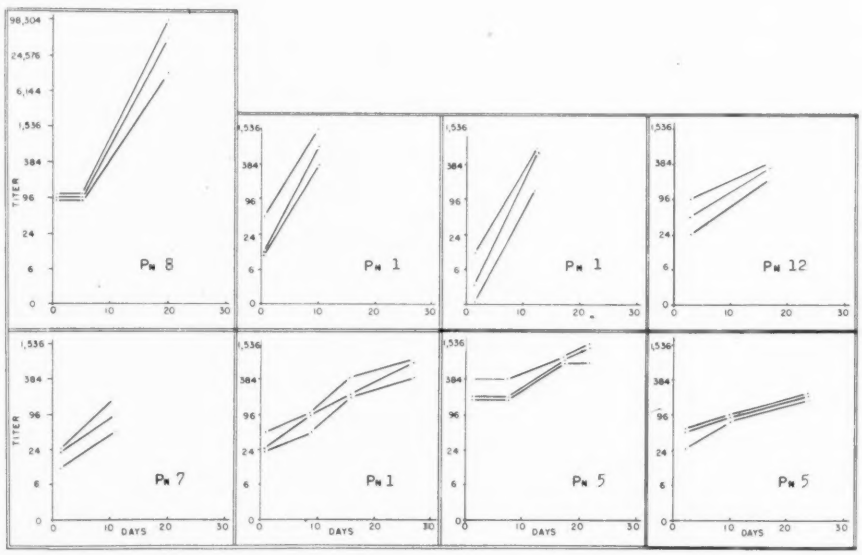

Fig. 3.—Antihyaluronidase in pneumococcic pneumonia; pneumococcus bacteremia with purulent complications.

three times, and sixty-four fold or greater seven times. From the curves in Fig. 3 it can be seen that all eight patients exhibited rises of titer which were fourfold or greater, and that four patients exhibited increases which were eightfold or greater. Rises of antihyaluronidase titer in six patients were apparent during the first week of hospitalization and in all patients were not specific for type of pneumococcus. In five patients who were observed for more than thirty days the highest rise of serum antihyaluronidase titer occurred ten to twenty-seven days after pneumococcus bacteremia.

The clinical data pertaining to these eight patients are shown in Table VII. The average age was 48.3 years. An average of 1.7 lobes was involved by pneumonia, and the average hospital stay was 41.0 days. The first six patients recovered; the seventh died of empyema on the twenty-fifth hospital day, and the eighth died of multiple lung abscesses on the thirty-second hospital day.

Particular Considerations of the Antihyaluronidase in the Serum of Patients With Pneumococcic Pneumonia: Studies which indicate that the antihyaluronidase in these sera was specifically antagonistic to hyaluronidase elaborated by the species pneumococcus have been reported elsewhere.[29]

TABLE VI. ANTIHYALURONIDASE IN PNEUMOCOCCIC PNEUMONIA; PNEUMOCOCCUS BACTEREMIA WITH PURULENT COMPLICATIONS; TABULATION OF SERUM ANTIHYALURONIDASE TITERS PLOTTED ON THE GRAPHS IN FIG. 3

PN. TYPE	DAYS ILL BEFORE ADMISSION	DAYS FROM ADMISSION TO FIRST SERUM	DAYS FROM FIRST SERUM TO HIGHEST TITER	TYPE OF PN. HYALURONIDASE ANTAGONIZED	TITER OF FIRST SERUM	TITER OF HIGHEST TITER
8	3	1	19	1	96	49,152
			48	2	96	196,608
			48	7	96	24,576
1	4	0	8	1	48	1,536
			31	2	12	1,536
			8	7	12	384
1	4	1	12	1	12	768
			12	2	3	768
			12	7	<3	192
12	9	3	47	1	96	384
			89	2	48	384
			14	7	24	192
7	Unknown	1	10	1	12	48
			10	2	24	96
			10	7	24	192
1	Unknown	1	26	1	48	768
			26	2	24	768
			26	7	24	384
5*	3	1	19	1	384	1,536
			16	2	192	768
			19	7	192	1,536
5†	7	2	22	1	48	192
			22	2	24	192
			22	7	48	192

*This patient died of empyema.
†This patient died of multiple lung abscesses.

TABLE VII. ANTIHYALURONIDASE IN PNEUMOCOCCIC PNEUMONIA; PNEUMOCOCCUS BACTEREMIA WITH PURULENT COMPLICATIONS; CLINICAL DATA

PN. TYPE	AGE OF PATIENT (YR.)	LOBES OF LUNG INVOLVED	TREATMENT*			COMPLICATIONS	DAYS IN HOSPITAL
			SULFA.	PENICILLIN	SERUM		
8	17	RL	+	0	0	Empyema	61
1	37	LL	+	+	+	Empyema	106
1	43	LL	+	0†	0	Empyema	45
12	71	RU	+	+	0	Pyarthrosis, azotemia	63
7	52	RU, RM, RL	+	+	+	Meningitis	41
1	52	RU, RM, RL	+	+	0	Empyema	61
5‡	55	RL	+	+	0	Empyema	25
5‡	60	LL, RU, RL	+	+	0	Lung abscesses	32
Average	48.3	1.75					41.0

*Sulfa., Ordinary doses of sulfamerazine[1] or sulfadiazine; penicillin, 20,000 to 25,000 units of amorphous penicillin every three hours intramuscularly; serum, type-specific therapeutic antipneumococcic rabbit serum (Lederle).
†This patient received intrapleural penicillin only.
‡These patients died on the twenty-fifth and thirty-second hospital day respectively.

Therapeutic antipneumococcic rabbit serum exhibited very little antihyaluronidase. The highest dilution of Type 2 concentrated antipneumococcic serum* which antagonized the constant amount of Type 2 pneumococcus hyaluronidase was 1:12.

*The antisera tested were Lederle Lot No. 472H676J, 50,000 units per vial, and Lilly, Lot No. B4555, 20,000 units per vial.

Intravenous administration of 200,000 units of Type 1 therapeutic antipneumococcic rabbit serum (Lederle) in two injections to a patient with Type 1 pneumonia did not bring about an increase of the patient's serum antihyaluronidase titer in the two-day period following the antiserum but did produce a marked rise in titer of agglutinins for the specific pneumococcus. There were no agglutinins for Type 1 pneumococcus before the antiserum, but there was a progressive rise of agglutinin titer to 1:2,560 (2 plus) as observed at four intervals in the two-day period. One patient with Type 7 pneumonia who was given 200,000 units of Type 7 antipneumococcic rabbit serum intravenously in one injection exhibited no change of serum antihyaluronidase titer twenty days later.

High therapeutic concentrations of penicillin and sodium sulfadiazine did not exhibit antihyaluronidase activity when titrated against pneumococcus hyaluronidase. The concentrations tested were 4 units of penicillin per cubic centimeter (Food and Drug Administration Standard) and 20 mg. per 100 c.c. of sodium sulfadiazine.

Low titer and high titer antihyaluronidase sera from one patient and sera from two other patients exhibited no loss of antihyaluronidase activity following one hour at 56° C. in a water bath.

Patients with other pneumococcus infections without pneumonia exhibited rises of serum antihyaluronidase titer. One patient with pneumococcic meningitis secondary to mastoiditis and another patient with mesothelioma of the pleura complicated by pneumococcic empyema exhibited, respectively, fourfold and eightfold rises of serum antihyaluronidase titer as tested with the three pneumococcus hyaluronidases. One patient with Type 12 pneumococcic aortic endocarditis and no evidence of pneumonia exhibited a rise of serum antihyaluronidase titer from the titer of 1:3 to the titer of 1:192 as titrated with Type 2 pneumococcus hyaluronidase.

DISCUSSION

The rises of serum antihyaluronidase titer reported here indicate that hyaluronidase is elaborated in the bacteremic pneumococcic pneumonias. In general, following an optimal injection of an antigen there is a lag of a few days before the appearance in the blood of the corresponding antibody. The titer of this antibody rises rapidly to a maximum which usually is attained between the tenth and twenty-second day after inoculation.[28] According to these principles, elaboration of hyaluronidase in fourteen of sixteen cases of bacteremic pneumococcic pneumonia began during or before the first week of hospitalization.

The relation of this elaboration of hyaluronidase to the severity of pneumococcic pneumonia is not clear. The rises of serum antihyaluronidase titer which were exhibited by all patients with purulent complication suggest that hyaluronidase may promote increased permeability of pleural, meningeal, or synovial tissues. In corroboration of this impression, all patients whose serum exhibited no increase of antihyaluronidase titer were free of purulent complica-

tion. Increase of antihyaluronidase titer could not be correlated with the number of lobes involved by the pneumonia since the average number of lobes involved was similar in all groups.

The antihyaluronidase reported here may be similar to the nonantibacterial therapeutic factor which was reported to be present in antipneumococcic horse serum by Sabin in 1932.[27]

The occurrence of small amounts of antihyaluronidase antagonistic to pneumococcus hyaluronidase in the serum of all of fifty normal human beings is notable, but its significance is obscure. Ten of these people were high school youths 17 and 18 years of age, and forty were medical personnel of the Cincinnati General Hospital between 20 and 30 years of age.

The absence of type specificity of the antihyaluronidase response in pneumococcic pneumonia is consistent with the finding of Friou and Wenner[26] that an inhibitory substance in the serum of rabbits immunized with pneumococci neutralized enzymes (hyaluronidases) derived from six different types of pneumococci.

SUMMARY

Antihyaluronidase in the serum of fifty normal human beings antagonistic to Type 2 pneumococcus hyaluronidase was compared with the same antihyaluronidase in the serum of Patient A. T. who was six months convalescent from Type 2 pneumococcus endocarditis. All of the fifty normal subjects tested had some degree of this antihyaluronidase. Since subsequent experiments demonstrated that serum antihyaluronidase antagonistic to pneumococcus hyaluronidase is not type specific, these observations upon the sera of normal human beings pertain to hyaluronidase elaborated by all types of pneumococci.

Serial sera of twenty-six patients with primary pneumococcic pneumonia were tested for antihyaluronidase by titration against hyaluronidases in culture filtrates of Type 1, Type 2, and Type 7 pneumococci. Seven patients with negative blood culture and no purulent complication exhibited no change of antihyaluronidase titer. Eight of eleven patients with pneumococcus bacteremia and no purulent complication exhibited rises of antihyaluronidase titer which were fourfold or greater. All of eight patients with pneumococcus bacteremia and purulent complication exhibited rises of titer of fourfold or greater. The rises of antihyaluronidase titer which were observed were not specific for pneumococcus type.

Therapeutic antipneumococcic rabbit serum exhibited less antagonism of pneumococcus hyaluronidase than did a majority of the sera from fifty normal human beings. High therapeutic concentrations of penicillin and sodium sulfadiazine exhibited no antagonism of pneumococcus hyaluronidase.

A rise of serum antihyaluronidase titer could not be brought about in one patient with pneumococcic pneumonia by the intravenous administration of therapeutic antipneumococcic serum, but a progressive rise of serum agglutinin titer for the homologous pneumococcus was produced.

CONCLUSION

Rises in the titer of serum antihyaluronidase antagonistic to pneumococcus hyaluronidase, found in sixteen patients with bacteremic pneumococcic pneumonia, indicate that hyaluronidase was elaborated and was antigenic in these pneumococcus infections. This elaboration of hyaluronidase was apparent during the first week of hospitalization in fourteen of these sixteen patients with pneumonia. The elaboration of hyaluronidase appears to be related in some way to the development of purulent complications in pneumococcic pneumonia.

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THE ROLE OF HIGH BLOOD PENICILLIN LEVELS ACHIEVED WITH CARONAMIDE IN PENETRATING THE BLOOD-BRAIN BARRIER

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IN A previous report[1] we described a method for producing sustained high penicillin levels in the blood. This consisted of frequent, rapid intravenous injections of large doses of crystalline penicillin in conjunction with the oral administration of Caronamide (4′-carboxyphenylmethanesulfonanilide). The latter inhibits tubular excretion of the antibiotic.[2] The method was found to be safe and effective.

In a review of previous investigations, Wyke[3] reported disagreement on the question of whether any penicillin traverses the blood-brain barrier under normal and abnormal conditions. When penetration into the spinal fluid was reported the levels recorded were low. Smith and collaborators[4] gave 100,000 units of penicillin intravenously to patients with normal and inflamed meninges and found only traces of penicillin in the spinal fluid. They concluded that the meninges are relatively impermeable to penicillin. Schwemleim and co-workers[5] administered penicillin by intravenous drip in amounts varying from 10 to 25 million units in twenty-four hours and found the highest spinal fluid level thus achieved to be 0.55 Oxford unit per milliliter. Because of these and similar findings by other authors, cerebrospinal infections have been treated by the intrathecal route, a procedure not without hazard.[6-8]

For the study reported below, twelve patients free of infection of the central nervous system were selected. In eight of these twelve patients, one million units of crystalline penicillin were administered rapidly intravenously every hour for ten hours. In four, namely Patients 3, 4, 5, and 7, a continuous intravenous infusion of 10 per cent glucose in 1,000 c.c. of distilled water was administered during the ten-hour period, and the penicillin was injected every hour into the distal end of the rubber tubing. Eight patients received Caronamide in doses of 4 Gm. every three hours starting eighteen hours prior to and continuing during the period of administration of the antibiotic. These included six patients who received the hourly injections and two who received penicillin along with a continuous intravenous injection of glucose. For comparison Caronamide was withheld from the remaining four patients.

The peripheral venous blood and the spinal fluid samples were obtained approximately fifteen minutes after the last (tenth) injection of the penicillin. In some of the patients specimens also were obtained fifteen minutes after the fifth as well as the tenth injection, and fourteen hours after the last (tenth) injection.

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The method of penicillin assay was a broth tube dilution method using *Staphylococcus aureus*, strain H, as the standard organism and fresh meat extract broth as the medium. The minimal concentration of the standard penicillin* required to inhibit the inoculum of 5×10^2 *Staph. aureus*, strain H, cells was 0.02 unit per milliliter. All titrations of blood and spinal fluid levels were accompanied by and compared with this standard.

TABLE I. PENICILLIN LEVELS IN THE BLOOD AND CEREBROSPINAL FLUID AFTER THE ADMINISTRATION OF ONE MILLION UNITS OF CRYSTALLINE PENICILLIN EVERY HOUR FOR TEN HOURS, WITH AND WITHOUT CARONAMIDE (IN OXFORD UNITS PER MILLILITER)

	TIME					
	AFTER FIFTH INJECTION		AFTER TENTH INJECTION		14 HOURS LATER	
PATIENT	BLOOD	SPINAL FLUID	BLOOD	SPINAL FLUID	BLOOD	SPINAL FLUID
			With Caronamide			
1	266.0	2.0	160.0	4.4	2.0	0.8
2	260.0	2.0	280.0	6.0	4.4	0.8
3*			100.0	2.0		
4*			140.0	5.0		
5			280.0	1.0		
6			280.0	6.6	20.0	0.08
7			220.0	4.4		
8			300.0	2.5		
			Without Caronamide			
3			40.0	0.24		
5*			12.5	0.133		
7*			40.0	1.0		
9			20.0	0.133		

*Injection made into tubing of continuous intravenous drip.

It may be seen from Table I that substantial penicillin levels were achieved in the cerebrospinal fluid, especially when Caronamide was employed as an adjuvant. The levels in this group ranged from 1.0 to 6.6 units per milliliter of spinal fluid, with an average of 4.0 units per milliliter. In two patients tested after the fifth injection a level of 2.0 units of penicillin per milliliter of spinal fluid was present. Penicillin was still found in the spinal fluid in the patients tested fourteen hours after the last injection.

In a number of instances the blood penicillin levels were not as elevated as had been anticipated. This occurred in two patients who received the antibiotic via the intravenous tubing through which a 10 per cent solution of glucose was being administered. It is believed that such factors as dilution and loss of penicillin because of diuresis may have played a role. In all the patients who received intermittent injections and Caronamide, the blood penicillin levels were markedly elevated as previously reported.[1] Considerably less penicillin was found in the blood and spinal fluid of those patients who did not receive Caronamide.

These studies demonstrate that substantial penicillin levels can be attained in the cerebrospinal fluid of patients with normal cerebrospinal systems. These levels are presumably due to the elevated blood levels and not to a change in permeability of the meninges. However, it is possible that even higher levels

*Obtained from the United States Department of Agriculture.

might be attained in the presence of diseased and more permeable membranes. In such cases intrathecal administration of penicillin in the treatment of the infections might prove unnecessary.

SUMMARY

High penicillin levels in the cerebrospinal fluid were produced in normal individuals by a method designed to produce high, sustained blood levels. In three instances the presence of the antibiotic was still demonstrable in the spinal fluid fourteen hours after the last intravenous injection of penicillin.

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THE EFFECT OF SODIUM SALICYLATE UPON SERUM DISEASE IN RABBITS

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INTRODUCTION

THE therapeutic value of salicylates in the treatment of acute rheumatic fever and serum sickness has long been recognized,[1, 2] but most clinical experience has indicated that these drugs have no specific effect upon the disease processes.[1, 3-5] There has been little opportunity however to observe whether the lesions of these diseases are altered by salicylate therapy. Although an experimental approach would be helpful in evaluating the histopathologic effects of these drugs, in the past this type of study has been impossible because of the absence of a suitable counterpart of rheumatic fever or serum sickness in animals. Recently such an opportunity has been offered by the production of serum disease in rabbits[6] and the demonstration that it is characterized by lesions of the arteries and heart which are similar in several respects to those of acute rheumatic fever.[7-9]

The following experiments were undertaken, therefore, to test the influence of sodium salicylate therapy upon the lesions resulting from the injection of rabbits with large intravenous doses of normal horse serum. Because a previous study had suggested that the necrotizing arteritis which some rabbits develop following horse serum injection might be due in part to arterial spasm,[10] this opportunity was taken to study the arterial blood pressures of some of the animals during the course of development of the lesions. In addition the erythrocyte sedimentation rates of the rabbits were measured, since preliminary observations indicated that they became elevated during the course of serum disease in the rabbit as in man. It was hoped that these determinations might give a clinical measure of the severity of the developing lesion where other criteria had failed.[10] We also wished to ascertain the effects, if any, of salicylate therapy upon the increased sedimentation rates.

EXPERIMENTAL PROCEDURE AND MATERIALS

Two experiments were performed. In the first, fifteen rabbits were used. During a preliminary period of eight days before the first injection of horse serum, four determinations of each animal's blood pressure and sedimentation rate were made to establish normal values. On the first day of the experiment all but two of the rabbits were injected with 10 ml. per kilogram of horse serum intravenously. The two uninjected animals served as controls, one for the blood pressure determinations and the other for salicylate therapy. Arterial blood pressures and erythrocyte sedimentation rates, the latter always accompanied by cell volume

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determinations, were measured on alternate days throughout the experiment. On the sixth day following the initial injection of horse serum a number of the rabbits began to show elevated sedimentation rates and erythematous ears[6] suggesting a developing serum reaction. Sodium salicylate treatment was then begun in half the injected rabbits and one of the uninjected controls. In the treatment of acute rheumatic fever Coburn advocated maintaining plasma salicylate levels above 350 gamma per milliliter. Because this average level in rabbits almost always results in severe toxicity, the drug was administered in quantities designed to maintain an average of about 250 gamma per milliliter throughout the twenty-four hour period. The animals were paired so that those showing similar sedimentation rate increases were divided between the salicylate treated and the untreated groups. Salicylate therapy was continued through the tenth day when it was assumed that the tissue reaction to the first injection of horse serum was complete.[6] On the sixteenth day of the experiment, salicylate therapy was resumed in the treated rabbits. Since in our experience arteritis has been produced in greater intensity and in more animals following a second large injection of horse serum, all of the animals were reinjected intravenously on the seventeenth day of the experiment with 10 ml. per kilogram. Salicylate therapy was continued for the following eleven days, at the end of which time the treated rabbits and their controls were sacrificed and suitable sections were taken for histologic study.

Twenty-two rabbits were used in the second experiment which was similar in plan to the first with the following exceptions: Blood pressures were not determined. Sedimentation rates and hematocrits were each measured twice before the experiment started, five, seven, nine, twelve, and fourteen days after the first injection of horse serum and two, four, and six days after the second injection. Eighteen rabbits were injected with horse serum, three received salicylate only, and one served as a control on the effects of bleeding. Ten rabbits of the group injected with horse serum were treated with salicylate beginning on the sixth day, and treatment was continued in the survivors until the experiment ended. A second injection of horse serum was given on the sixteenth day of the experiment and the survivors were sacrificed on the twenty-second day.

Animals.—Thirty-seven adult rabbits of both sexes were used. With the exception of four animals they were all albinos. Their initial weights varied from 1.9 to 3.9 kilograms. They were caged singly and fed a commercial rabbit food* and water ad libitum.

Horse Serum.—Two lots of normal horse serum without preservative were used,† one for each experiment. An aliquot of each was cultured immediately before it was injected and was found to be sterile. The serum was injected slowly into a lateral ear vein using sterile precautions.

Sedimentation Rates.—These were determined using thick-walled capillary tubes with the specifications of Westergren.[12] The tubes were filled to a 20 cm. mark and their lower ends were plugged with modeling clay. They were kept in a vertical position by the method suggested by Foster.[13] Dry oxalate mixture[14] served as the anticoagulant. Hematocrits were always determined simultaneously by a micromethod.[15] The sedimentation rate readings were made at two hours and were corrected for anemia with data obtained in this laboratory by Lushbaugh[16] who employed this technique with the same strain of rabbits. With this method the healthy rabbit with a normal hematocrit has a two-hour sedimentation rate of less than 5 millimeters.

Arterial Blood Pressures.—These were measured using the method and apparatus described by Grant and Rothschild.[17] The same point on the same ear artery was used for each determination. The ear was always warmed until there was maximal vasodilatation in front of the light which served as the source of illumination for the readings. Then three readings were made in rapid succession and the average was recorded as the blood pressure. Both systolic and diastolic readings were obtained.

Sodium Salicylate Administration.—Preliminary experiments indicated that it was necessary to give a total quantity of 750 mg. per kilogram in divided doses each day in order to maintain salicylate levels of approximately 250 gamma per milliliter or above throughout most

*Rockland Rabbit Pellets.

†Kindly supplied by Dr. F. G. Jones of the Lilly Research Foundation, Indianapolis, Ind.

of the twenty-four hour period. In the first experiment the salicylate was administered to each treated rabbit in three divided doses of 250 mg. per kilogram at 8:30 A.M., 4:30 P.M., and 10:30 P.M. Two routes of administration were tried in this experiment. On days seven, eight, and nine a 5 per cent solution of sodium salicylate in distilled water was fed by stomach tube. Because of technical difficulty in passing the tube and the death of one of the rabbits from tracheal passage, the drug was given subcutaneously in a 4 per cent solution on the tenth day. When therapy was resumed on the sixteenth day the subcutaneous route was again used and continued until the twenty-eighth day. An occasional dose was omitted if the animal appeared toxic.

In the second experiment, a 5 per cent aqueous solution of sodium salicylate was given by stomach tube throughout the treatment period. This route was chosen because further observations had indicated that a more sustained level could be attained in this fashion and further experience had made this route safer. Although the times of administration were similar to those of the first experiment, the amount of drug was varied from rabbit to rabbit and from day to day as follows: A total of 850 mg. per kilogram divided into three doses was given for the first twenty-four hours. Thereafter the daily quantities were adjusted according to the plasma levels obtained in an attempt to maintain comparable levels. The divided doses varied between 75, 75, and 100 mg. per kilogram per day and 300, 300, and 500 mg. per kilogram per day. The larger amount always was given at 10:30 P.M. in an attempt to maintain a high level over the ten-hour period at night.

Plasma Salicylate Determinations.—These were made according to the colorimetric method of Brodie, Udenfriend, and Coburn.[18] Readings were taken with a Klett-Summerson photoelectric colorimeter using a filter with a wave length of 540 millimicrons. On the days when determinations were made on the treated rabbits, an equivalent amount of blood was drawn from the untreated rabbits in order to equalize blood loss.

Antibody Titers.—Serum was collected on the sixteenth day in the first experiment and on days seven, fourteen, and twenty-one in the second experiment from all the rabbits. This was stored in paraffin sealed tubes at −20° C. until the end of the experiment when precipitin titers to horse serum were determined by the collodion particle agglutination technique of Cannon and Marshall.[19]

Histologic Studies.—The animals which survived were sacrificed by air embolism. In the first experiment a single transverse section through the base of the heart was made; in the second, four sections of the heart were made, one through each of the four valve rings. In addition, in each experiment sections of the lungs, mediastinum, diaphragm, liver, stomach, pancreas and spleen, mesenteric lymph nodes, left kidney, left adrenal, bone marrow, and testis or uterus and ovary from each rabbit were examined. All tissues were fixed in Zenker-formalin solution, embedded in paraffin, and stained with hematoxylin and eosin.

EXPERIMENTAL RESULTS

Sedimentation Rates.—Fig. 1 shows the corrected two-hour erythrocyte sedimentation rates and the mean rates found in the treated and untreated groups in the second experiment. The changes in the sedimentation rates in the two experiments were so similar that only those of experiment two are given in detail. Normal rates persisted in the salicylate treated and bleeding control animals which did not receive intravenous horse serum. Of the thirty-one animals given horse serum only five failed to show a rise above 20 mm. after the first or second injection. The rate usually began to rise from the fifth to the seventh day, at the time when the first manifestations of hypersensitivity were seen. A further elevation usually occurred one to two days after the second injection of horse serum when presumably the extent and severity of the lesions were increasing. Salicylate treatment did not exert any notable effect on the sedimentation rate in spite of the fact that the lesions were less severe in this group of animals.

No lesions except those attributable to hypersensitivity could be found at autopsy to account for the observed changes of the sedimentation rate.

It should be noted that all of the rabbits showed a decreased cell volume. The average hematocrit of twelve salicylate treated animals fell from 42.0 to 25.8 per cent, while the average of twelve untreated rabbits fell from 41.0 to 30.7 per cent. Similarly, the hematocrits of the rabbits receiving salicylate alone dropped about twice as much as those of the bleeding controls. It appears that salicylate in this dosage increases the anemia which rabbits receiving large doses of horse serum develop.

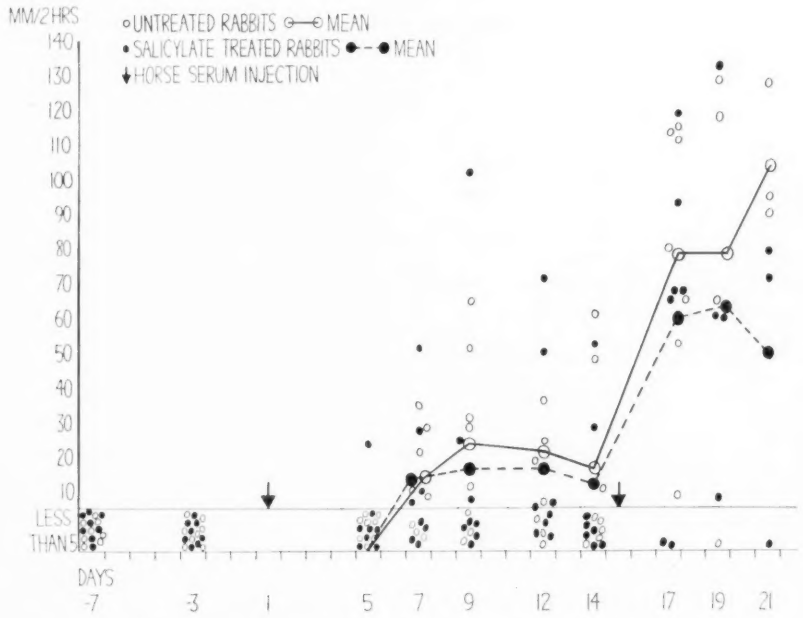

Fig. 1.—Erythrocyte sedimentation rates of rabbits injected with horse serum.

Blood Pressure Findings.—For purposes of comparison the nine rabbits which received two injections of horse serum and survived to the end of the first experiment were divided into three groups (Fig. 2). Group A consisted of three rabbits which at death manifested severe arteritis; Group B was composed of three similarly injected rabbits which developed minimal or no arteritis, and Group C, the three rabbits which received both serum and salicylate and which also showed only slight arterial reaction. Only the systolic pressures are recorded in the graph since the changes in the diastolic pressures were parallel.

Sustained hypertension was not noted in any animal of these three groups. It is of interest however that there was a definite trend for the arterial pressures of the untreated groups (A and B) to rise following the first injection of horse serum. However this was no more marked in the rabbits which later exhibited lesions than in those which reacted with little or no arteritis. This elevation was most evident from day seven to day eleven following injection, an interval cor-

responding to the manifestations of serum disease in the rabbit, namely ear erythema,[6] temperature rise,[20] and rise in sedimentation rate (Fig. 1). On the other hand the treated rabbits which were receiving salicylate during this period (day seven through day ten) showed a sharp drop in blood pressure. When salicylate therapy was stopped there was a rise toward the preinjection level.

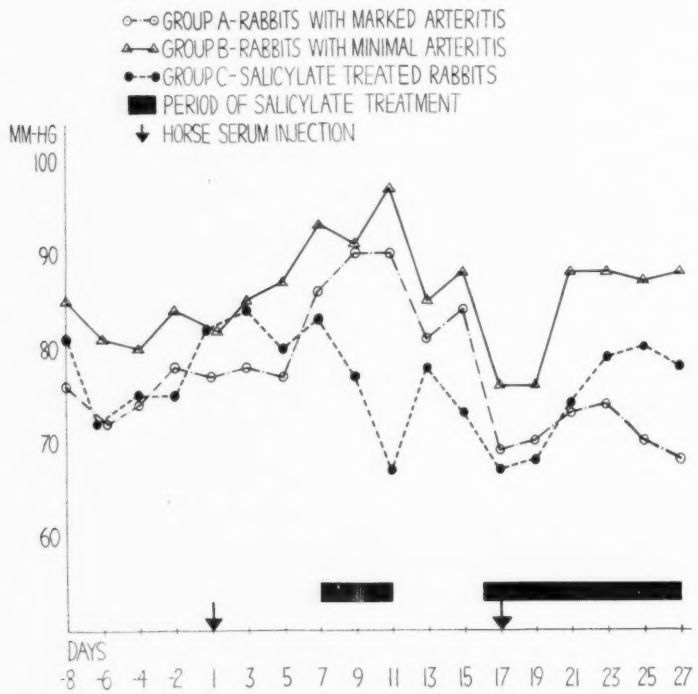

Fig. 2.—Mean systolic blood pressures of rabbits injected with horse serum.

The second injection of horse serum on the seventeenth day was accompanied by a fall in the arterial blood pressure which was most apparent in the two untreated groups, A and B. The remainder of the observation period was characterized by a gradual rise of the arterial pressure in Groups B and C to values comparable to the preinjection levels (Group C) or slightly higher (Group B). The rabbits which were developing marked generalized arteritis (Group A) showed no significant change in their blood pressure from day nineteen to day twenty-seven. Furthermore the drop in blood pressure observed in the salicylate treated group in the first treatment period did not recur during this course of therapy.

Salicylate Levels.—Maintenance of adequate salicylate levels was difficult because of wide variation in the animals' tolerance for the drug. In spite of careful adjustment of individual doses, seven rabbits died of salicylate intoxication before the completion of the experiments. The level was considered to be adequate during most of the twenty-four hour period if a plasma concentration of 150 gamma per milliliter or more was present five hours after the previous administration of the drug. In the first experiment plasma salicylate levels were

determined on days ten and eighteen at six hours and on the twentieth day at three hours after the morning dose. These levels were always above 150 gamma and the average of the three determinations in each animal ranged between 343 and 544 gamma. In the second experiment plasma salicylate concentrations were measured on the eighth day twelve hours after the evening dose of drug and on days nine, twelve, fourteen, sixteen and nineteen five hours after the morning dose. Individual values ranged between 0 and 530 gamma. In four animals, average five-hour levels were between 254 and 353 gamma. The other four animals had average five-hour readings of 90 to 128 gamma but were receiving such large amounts of salicylate (0.9 to 1.1 Gm. per kilogram per day) that it did not seem wise to increase the dose further.

Histopathologic Findings.—Table I summarizes the findings in experiment one in four treated and four untreated rabbits which were paired on the basis of similar responses in sedimentation rate following the first injection of horse

TABLE I. EXTENT AND SEVERITY OF THE LESIONS OF SERUM DISEASE IN TREATED AND UNTREATED PAIRS OF RABBITS (EXPERIMENT ONE)

HORSE SERUM AND SODIUM SALICYLATE					HORSE SERUM				
		HISTOLOGIC LESIONS					HISTOLOGIC LESIONS		
RABBIT	DAY OF DEATH	MYOCARDIAL	ARTERIAL	RENAL	RABBIT	DAY OF DEATH	MYOCARDIAL	ARTERIAL	RENAL
7	17	0	0	0	4	17	0	+++	0
2	28	0	+	0	13	28	0	++++	±
6	28	0	++	±	1	28	0	++++	0
8	28	0	0	0	14	28	0	±	0

serum. One pair died of an anaphylactic shock immediately following the second injection of horse serum, and the other three pairs survived to the end of the experiment. The lesions were graded separately by two observers from 1 plus to 4 plus depending upon their extent and severity. The extent of the valvular lesions could not be evaluated in this experiment because of the manner in which the heart sections were made. It should be noted that the numbers of animals to be compared were small in this experiment because two salicylate treated rabbits died on days eight and nine. Despite the small series, the results strongly suggest that salicylate treatment had depressed the developing arteritis in the salicylate treated group.

TABLE II. EXTENT AND SEVERITY OF THE LESIONS OF SERUM DISEASE AND PRECIPITIN TITERS TO HORSE SERUM AT DEATH IN TREATED AND UNTREATED PAIRS OF RABBITS (EXPERIMENT TWO)

HORSE SERUM AND SODIUM SALICYLATE							HORSE SERUM						
		HISTOLOGIC LESIONS							HISTOLOGIC LESIONS				
RABBIT	DAY OF DEATH	VALVULAR	MYOCARDIAL	ARTERIAL	RENAL	ANTIBODY TITER	RABBIT	DAY OF DEATH	VALVULAR	MYOCARDIAL	ARTERIAL	RENAL	ANTIBODY TITER
45	15	0	0	0	0	–	58	15	+++	0	++	+	–
59	18	0	0	0	0	–	55	18	++	0	0	0	1:160
60	18	0	+++	0	0	1:160	56	18	++++	++++	+	±	1:640
53	19	±	0	0	0	1:80	54	19	±	+++	0	0	1:160
47	19	++	0	+	0	1:160	48	19	+++	+++	++	+	1:1280
41	22	±	0	0	0	1:160	49	22	++	0	+	+	1:2560
42	22	±	0	±	0	1:640	46	22	++	0	++	0	1:2560
57	22	±	0	±	0	1:640	43	22	±	0	+	0	1:320

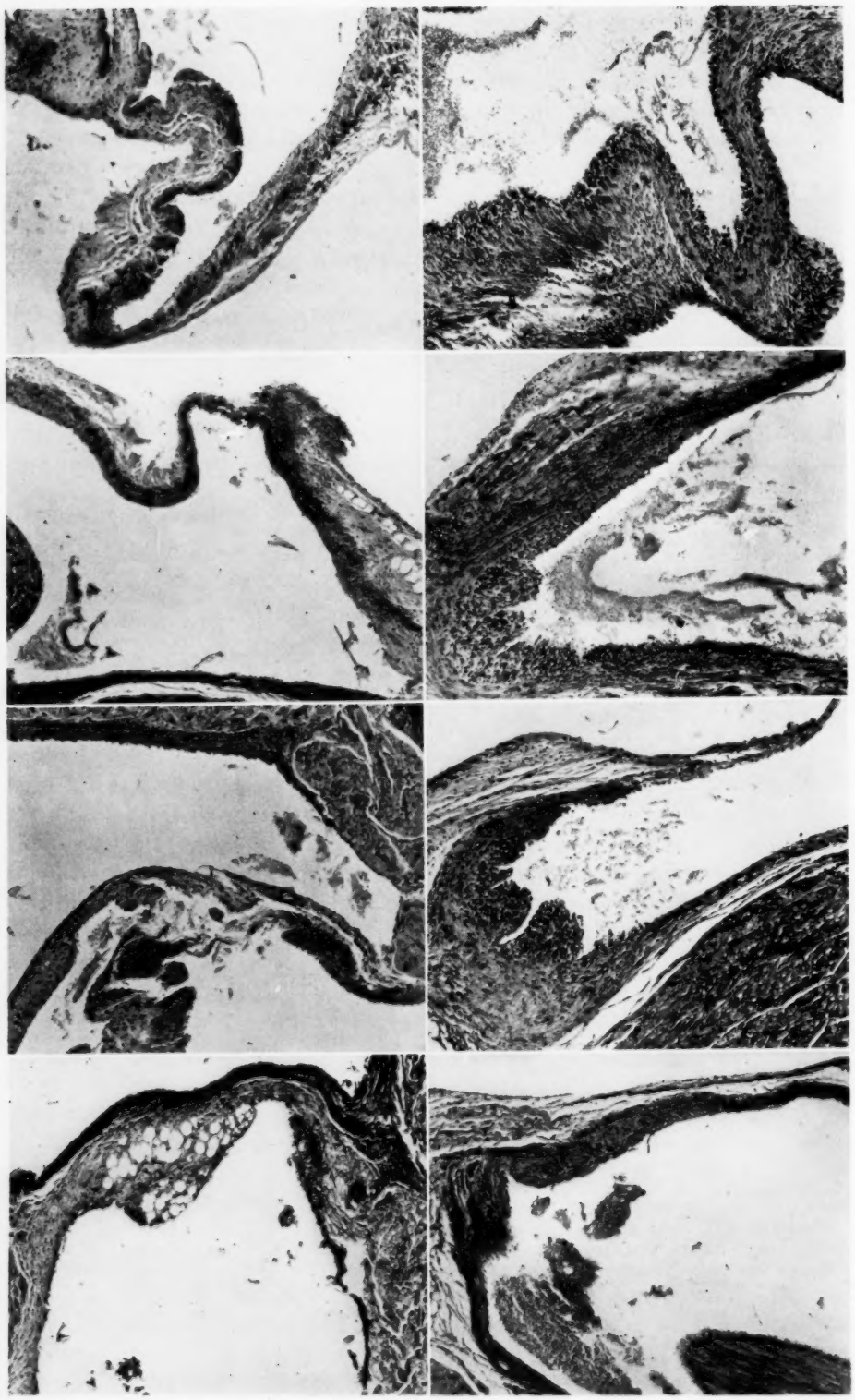

Fig. 3.—Mitral valves of rabbits treated with sodium salicylate (Rabbits 45, 59, 60, and 53) compared with their untreated controls (Rabbits 58, 55, 56, and 54). Magnification ×125.

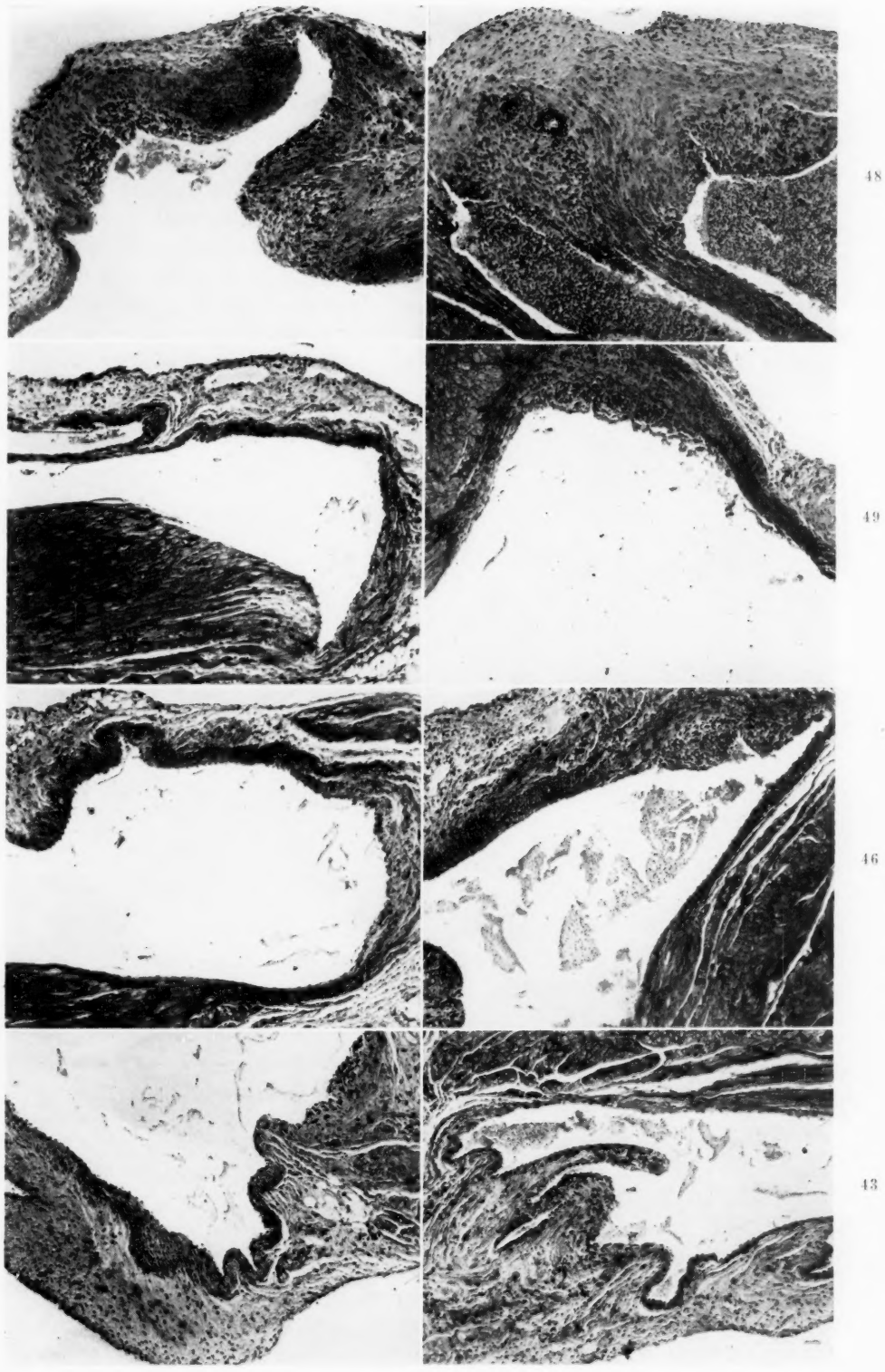

Fig. 4.—Mitral valves of rabbits treated with sodium salicylate (Rabbits 47, 41, 42, and 57) compared with their untreated controls (Rabbits 48, 49, 46, and 43). Magnification ×125.

Table II summarizes for experiment two the histologic findings in eight treated rabbits paired with eight untreated rabbits on the basis of the rise in the sedimentation rate after the first injection of horse serum. Two salicylate treated rabbits which had been injected with horse serum died early, one of false passage of the stomach tube and one of salicylate intoxication. These were eliminated from the experiment. Five salicylate treated animals died later, one of false passage of the stomach tube and four of salicylate intoxication. One died on day fourteen, two on day eighteen, and two on day nineteen. In each instance the paired untreated control animal was sacrificed on the same day.

It may be seen from Tables I and II that the lesions of the salicylate treated rabbits were considerably less severe and extensive than those observed in the untreated group. This fact is further emphasized by Figs. 3 and 4 which illustrate the changes in the mitral valves in each pair of rabbits in experiment two. For reasons which are not clear there was a much greater degree of necrotizing arteritis in the animals of experiment one, while the lesions observed in the second experiment were more severe in the myocardium and heart valves.

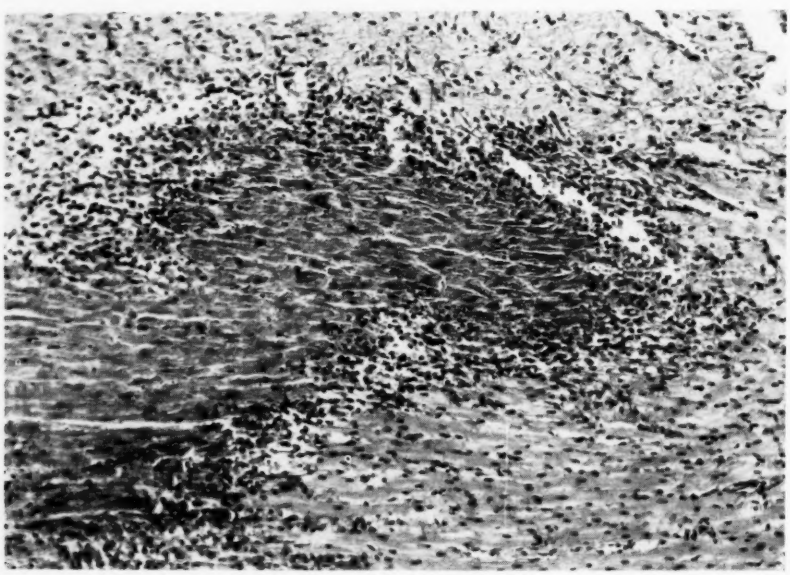

Fig. 5.—Area of necrosis in the right ventricle of Rabbit 60. Magnification ×140.

Microscopically the arteritis, when present, usually was seen in the medium sized arteries of the myocardium, lungs, pancreas, mesentery, stomach, kidney, liver, adrenal, diaphragm, and testis or uterus. Valvular changes, mostly proliferative in type, were found predominantly at the base of the valve leaflets. The latter were confined almost entirely to the mitral and aortic valves as they are in rheumatic fever. These lesions were all similar qualitatively to those described by Rich and Gregory.[7-9]

An additional lesion was noted in the myocardium; to our knowledge this has not been reported previously in rabbits with serum disease. The lesion con-

sisted of many areas of necrosis of varying size occurring almost exclusively in the myocardium of the right ventricle (Fig. 5). In no instance did we observe thrombosed arteries in the hearts which showed these lesions. Although the myocardial fibers in the areas were disrupted and undergoing necrosis, there was a notable absence of infiltration by inflammatory cells. In the older lesions there was early organization so that the whole area was composed of a network of large mononuclear cells. These appeared to be somewhat abnormal, large macrophages or young fibroblasts with clear chromatin-poor nuclei and large, deeply staining nucleoli (Fig. 6). The lesions were not seen in the animals which did not receive horse serum.

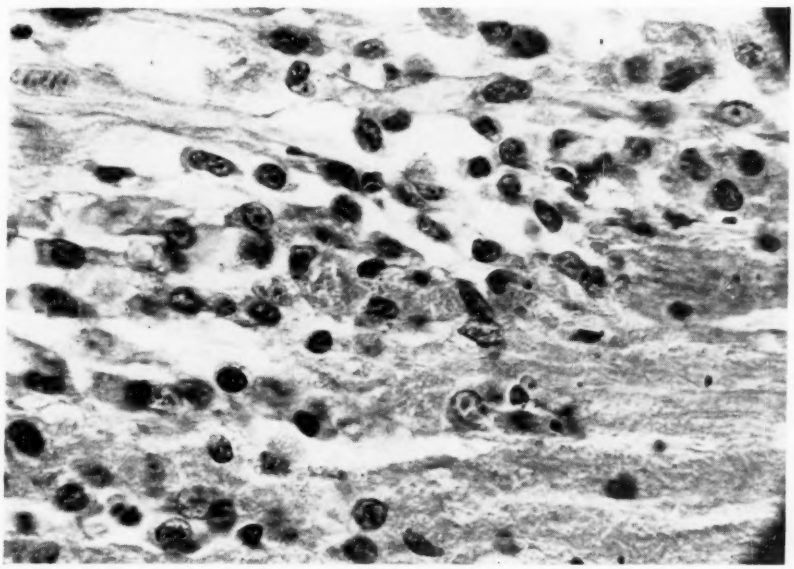

Fig. 6.—Higher magnification of an area at the edge of the lesion in Fig. 5. Magnification ×950.

The renal lesions usually were localized to the point where afferent arterioles entered glomeruli. At that point there was an apparent proliferation of endothelial cells or cells of the juxtaglomerular apparatus accompanied by a variable infiltration of lymphocytes. No significant changes were seen in the glomerular tufts. These lesions were mild and were observed in only a few animals (Tables I and II).

Except for pulmonary hemorrhage and edema and glycogen depletion of some of the livers, there were no changes in the tissues of the salicylate treated animals or the salicylate controls which could be attributed to toxic effects of the drug.

Antibody Titers.—Titers of sera obtained from the rabbits sixteen days after horse serum injection in experiment one and seven and fourteen days after injection in experiment two were similar in the treated and untreated groups. Appreciable differences in precipitin titers between the salicylate treated and untreated rabbits occurred in sera drawn eighteen to twenty-two

days after the first injection of horse serum in the second experiment (two to six days after the second injection). These are recorded in Table II where it can be seen that with one exception higher titers were recorded for the untreated member of each pair. The only pair of rabbits in which this difference was not observed (Rabbits 57 and 43) showed the least contrast in the severity and extent of their lesions.

DISCUSSION

The blood pressure observations do not support the concept that the arterial lesions develop as a result of arterial spasm[10] or that hypertension is the important common factor in the pathogenesis of experimental periarteritis.[21] This is in agreement with the experimental results of Hopps and McCollum[22] who failed to find a correlation between blood pressure changes and the lesions occurring in rabbits receiving large doses of horse serum. The transient elevation of blood pressure following the initial injection of horse serum might be interpreted as a manifestation of a generalized arterial constriction. Its inconstant occurrence following the second injection of serum and the lack of correlation of the rise with the development of arterial lesions make it untenable to assign to hypertension any significant role in the developing arteritis. On the other hand, the observations do not disprove the hypothesis that local arterial spasm may occur and be of some importance in the development of the arterial lesions. It is possible that the medial ear arteries utilized for the blood pressure determinations were unsuitable to reflect a generalized vasospastic reaction. For the present the question of this phase of the pathogenesis of these arterial lesions must be left unanswered.

The erythrocyte sedimentation rate appears to offer a clinical measure of the developing lesions of hypersensitivity although the extent and severity of the lesions found do not always correlate with the degree of rise in sedimentation rate. Contrary to the clinical findings of some observers,[5, 23, 24] sodium salicylate exerted little if any suppressive effect on the erythrocyte sedimentation response.

In these experiments an attempt was made to give the rabbits massive doses of salicylate comparable to those advocated by Coburn for human beings.[11] However, as had been noted previously in patients given large doses of salicylates,[25] the quantities necessary to maintain a high salicylate blood level vary widely. It is extremely difficult to maintain a sufficiently high level in the rabbit without producing severe toxic reactions which often terminate in death. It appears from the results that the salicylate blood levels were adequate in most instances although levels of 150 gamma or less were noted at some period of the twenty-four hours in many of the animals.

It is obvious that the severe toxic reactions which some of the rabbits exhibited as well as the anemia which most of them developed may have acted in a nonspecific fashion to prevent the development of the lesions. It should be noted however that the contrast between the lesions of the treated and untreated rabbits was not limited to those which showed toxic symptoms. Furthermore, when an animal died with salicylate intoxication it usually had been noticeably sick for only a few hours, whereas the lesions appeared considerably older. It

will be important, nevertheless, to ascertain whether the lesions of rabbit serum disease can be prevented or treated with doses of salicylate which do not produce toxicity.

At present one can only speculate as to how salicylates produce their effects. It has been reported that salicylates depress antibody formation.[2, 26-29] Other writers do not agree.[30, 31a] Our studies suggest that antibody formation was somewhat diminished in the salicylate treated group late in the experiment. However since the concentration of antibody seems to have little correlation with development of the lesions,[10] the significance of this finding is questionable. Recent work by Dorfman and co-workers[31b] confirms that of Guerra[31c] and suggests that sodium salicylate inhibits the spreading effect of hyaluronidase.

The question of whether salicylates merely suppress the symptoms or whether they exert a specific influence upon the lesions of rheumatic fever has been reopened recently by Coburn.[11] He has emphasized the possible specific action of large doses of salicylates in suppressing the rheumatic process and preventing the ensuing carditis. A few confirmatory studies have emphasized the need to administer the massive quantities of salicylate early in the course of the disease.[32-34] However, Keith and Ross[4] found that even early treatment with large doses of salicylate did not hasten restoration of the sedimentation rate or diminish the incidence of subsequent heart damage as compared with early treatment with small doses of the drug. Others have reported that the intensive salicylate therapy recommended by Coburn did not alter the inflammatory reaction of rheumatic fever in the joints[3] or heart[5] in patients of whom most had had rheumatic fever for four or more weeks before treatment. Similarly, Wégria and Smull[25] found the duration of rheumatic attacks to be unaffected by massive doses of salicylate but suggested that early treatment might have proved efficacious. At present, then, the clinical value of maintaining high salicylate levels in the treatment of acute rheumatic fever is still on trial. It appears however that if salicylates are to produce a specific effect upon the lesions of rheumatic fever they must be given early in the course of the disease.

In contrast to the findings of Thomas and Stringfield[35] our results show that sodium salicylate, given in large doses early in the course of serum disease in rabbits, reduces the extent and severity of the resulting lesions. It must be emphasized that in these experiments salicylate therapy was begun six days after the initial injection of horse serum, at a time when the lesions of hypersensitivity are presumably just beginning to develop.[36] It was continued throughout the period following the second injection of horse serum when it is assumed that the most marked tissue reactions occur. Because of its insidious onset the first attack of rheumatic fever rarely is seen by the clinician at a comparably early stage. However, Schlesinger[37] and Coburn and Moore[38] have reported success in suppressing the rheumatic process by prophylactic administration of salicylate following streptococcal infection in subjects with previous rheumatic episodes. Further experiments are indicated to determine how long following the injection of rabbits with horse serum the salicylate therapy can be initiated and still exert its suppressive effect upon the development of the lesions.

Many have called attention to the clinical and pathologic similarities between acute rheumatic fever and serum sickness,[39] and in our opinion rabbit serum disease represents the closest approximation to acute rheumatic fever yet produced experimentally. Despite these attractive similarities, our experience indicates that rabbit serum disease does not fulfill all of the rigid pathologic criteria listed by Gross and co-workers[40] for the experimental production of rheumatic fever. The Aschoff-like lesions are not numerous. Although their cellular components and location are typical they seldom show fibrinoid necrosis. Pericarditis is very rare and when present it is usually focal and minimal. Verrucae are uncommon in the valvular lesions and we have been unable to produce chronic valvular lesions like those in rheumatic fever by many repeated injections of horse serum over long periods of time.[41] Therefore at present one must be cautious in assuming that the inhibitory effect of salicylates upon the lesions of rabbit serum disease can be applied to the lesions of rheumatic fever.

SUMMARY AND CONCLUSIONS

The histopathologic lesions produced in twelve rabbits receiving two intravenous injections of sterile normal horse serum fifteen to sixteen days apart in doses of 10 ml. per kilogram have been compared with those produced in twelve similarly treated rabbits which also received large doses of sodium salicylate starting six days after the first injection of horse serum. Blood pressures were determined repeatedly in some of the rabbits (experiment one). Sedimentation rates and blood salicylate levels also were followed.

It is concluded that:

The arterial blood pressure of rabbits receiving large doses of horse serum showed no sustained elevation, and no evidence was obtained that a generalized arterial constriction plays a part in the pathogenesis of the arteritis of rabbit serum sickness.

The erythrocyte sedimentation rates of rabbits given large intravenous injections of horse serum appear to offer a clinical measure of the developing lesions of hypersensitivity. Although the degree of rise could be correlated to some extent with the severity of the lesions found, this correlation was not constant.

It is difficult to maintain salicylate levels of 350 gamma per milliliter recommended for man without producing intoxication and death in rabbits.

A moderate depression in the concentration of circulating antibody to horse serum was noted in the salicylate treated rabbits of the second experiment as compared with the untreated controls. This occurred eighteen to twenty-two days after the first injection of horse serum. Antibody concentrations earlier in the experiments were not significantly different in the treated and untreated groups.

A lesion of the right myocardium which occurred more frequently in the untreated rabbits is described. This consisted of focal necrosis accompanied by little inflammation.

The lesions seen in the salicylate treated rabbits, especially the arterial and valvular lesions, were greatly reduced in severity and extent as compared with those in the untreated animals.

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ALTERATIONS OF RADIAL OR BRACHIAL INTRA-ARTERIAL BLOOD PRESSURE AND OF THE ELECTROCARDIOGRAM INDUCED BY TILTING

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INTEREST in the response of blood pressure and pulse rate to postural change was stimulated by the problem of rapid evaluation of tachycardia in military induction examinations. Preliminary observations indicated that a significant slowing of heart rate occurred with tilting to a head-down position in subjects with tachycardia secondary to excitement. On the other hand, this slowing did not occur in inductees with tachycardia which was later found to be due to organic disease. An increase of blood pressure occurred in all subjects during the tilt to the head-down position. In this study careful analysis has been made of the response of heart rate and blood pressure to tilting in normal subjects in order to establish a standard to evaluate changed reactions due to disease or drugs.

Hill[1] first demonstrated the importance of gravity in the circulatory system of man. He also reported that the brachial systolic blood pressure, as measured by the sphygmomanometer, remained approximately the same on tilting from an erect to a head-down position, providing the readings were taken with the arm in the frontal plane perpendicular to the long axis of the body (horizontal plane). With the arm in this position the effect of gravity on the column of blood within the arteries of the arm remained constant.[2, 3] These readings were not taken during or immediately following postural change but after the subject was in the altered position for an appreciable period of time. Wald, Guernsey, and Scott,[4] using a Tychos self-recording sphygmomanometer, found that following a positional change from horizontal to standing the brachial blood pressure fell below the level obtained in the recumbent position in about ten seconds and then regained or exceeded this level within thirty seconds. Their results with tilting from standing to horizontal position varied in the two subjects studied. Other attempts to analyze the brachial arterial blood pressure changes with tilting have resulted in widely divergent findings.[5-9] There are two important explanations for the discrepancies in these reports. (1) It is impossible to obtain accurate readings of a rapidly changing blood pressure with a cuff method, and (2) the position in which the arm was maintained during the tilting has varied in the different experiments.

Slowing of the heart rate following a change from an erect to a horizontal or head-down position has been described by numerous observers.[5-8, 10, 11] However, these findings were interpreted from periodic pulse rate readings and the slowing was not consistently found in all normal subjects.

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In these studies we have recorded intra-arterial blood pressure continuously with the arm maintained in a horizontal plane. Heart rate and rhythm have been interpreted from an electrocardiogram taken throughout the experiment.

METHOD

Direct measurements of radial or brachial artery pressure during and after tilting have been obtained in more than two hundred subjects. This report details the findings in fifteen healthy young adults considered to have normal cardiovascular systems. Simultaneous electrocardiograms were obtained in these subjects, and in addition continuous electrocardiograms have been recorded in one hundred normal adults during and following tilting.

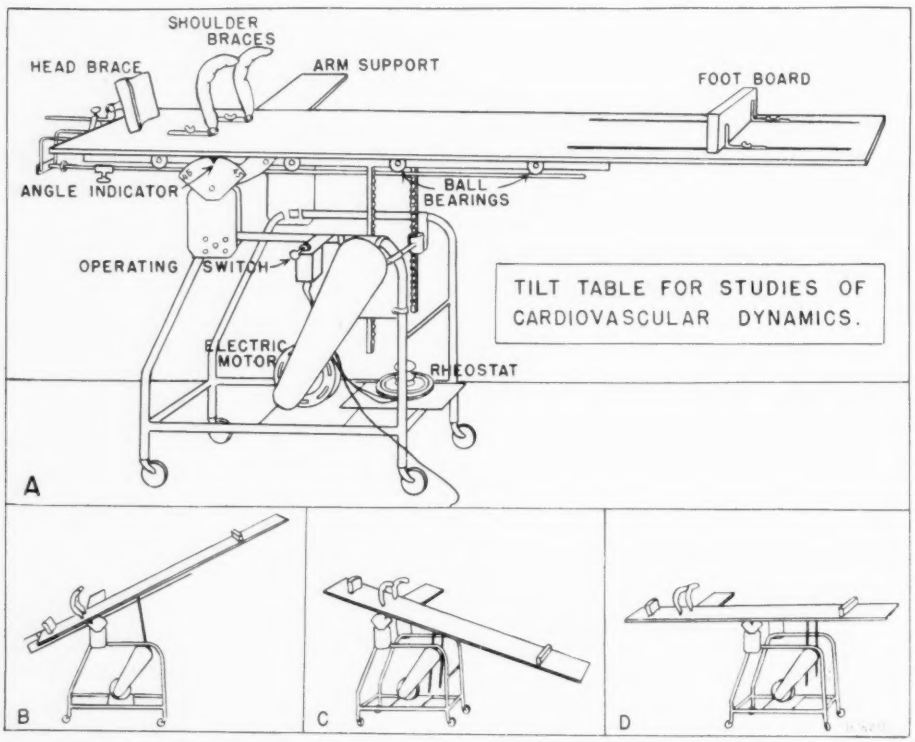

Fig. 1.—Tilt table for study of cardiovascular dynamics. *A*, *B*, *C*, Fulcrum at shoulder region: *A*, horizontal; *B*, 45 degree head-down position; *C*, 20 degree erect position. *D*, Fulcrum at iliac crest region.

The subjects were tilted at a moderate rate from the 20 degree head-up position to the 45 degree head-down position. This position was maintained for at least fifteen seconds. The subjects then were returned to the original position.

Tilting was controlled by means of an electrically operated table (Fig. 1). The subjects were securely supported so that muscular activity was not required to maintain the position.

The blood pressure tracings were determined by means of a Statham strain gauge adapted in our laboratory to record blood pressure changes by means of a string galvanometer electrocardiograph.*[12] The gauge was ideally suited to this type of experiment. It was

*Research Model Cambridge Electrocardiograph, Cambridge Instrument Co., Inc., New York, N. Y.

compact (3 by 1 by 1 inch) and could easily be held in place during movements. It did not respond to external vibration. Changes of pressure caused an alteration of electrical output that was transmitted to the recording string galvanometer. This output was directly proportional to the pressure; thus linear records were produced.

Preliminary investigation indicated that this system had adequate frequency for accurate pressure recording.

In these experiments a size 20, 1½ inch needle with a 45 degree sharp bevel was attached directly to the gauge. The system was completely filled with a solution of heparin (5 mg. per cubic centimeter), and the needle, with bevel directed toward the heart, was inserted into the brachial artery at the elbow or into the radial artery at the wrist.

At least three sets of observations were made on each subject.

RESULTS

Blood Pressure.—

Head-Down Tilt (20 Degrees Erect to 45 Degrees Head Down): An elevation of arterial blood pressure in the arm occurred during the head-down tilt in all of the subjects studied. In general the degree of rise was uniform throughout the tilt; the maximum blood pressure occurred immediately after cessation of motion.

In the subjects with normal cardiovascular systems this elevation of blood pressure (average rise, 19 mm. Hg systolic/16 mm. Hg diastolic) was followed by a gradual fall lasting from eight to eighteen seconds until the blood pressure reached a level that was usually slightly higher than that obtained in the erect position. The average fall in the normal subjects during this period was 13 mm. Hg systolic/14 mm. Hg diastolic (Figs. 2 and 3).

Head-Up Tilt (45 Degrees Head Down to 20 Degrees Erect): During the return tilt the blood pressure fell in all of the subjects. This decline was usually uniform throughout the tilt; the lowest blood pressure occurred immediately after the cessation of motion.

In the normal subjects this primary fall of blood pressure (average, 14 mm. Hg systolic/13 mm. Hg diastolic) reached a level below that initially obtained in the erect position, and it was followed by a rise; the blood pressure returned to the starting level within eight to eighteen seconds of the completion of the tilt (Figs. 2 and 3).

Heart Rate.—

Head-Down Tilt: In subjects with normal cardiovascular systems slowing of the heart rate invariably occurred subsequent to the elevation of blood pressure. The rate for the five-second period immediately following the onset of slowing was always less than the erect rate and less than 90 per minute (Figs. 2, 3, and 4). In the majority of cases the onset of slowing was abrupt, resulting in bradycardia which was marked for a few beats, the rate then gradually increasing. In others the slowing developed more gradually. Regardless of the type of the initial response the rate became relatively stable within about fifteen seconds of the completion of the tilt at a rate that was usually slower than previously recorded in the erect position.

Head-Up Tilt: The heart rate suddenly increased following the drop in blood pressure. This resulted in a rate that was more rapid than the initial

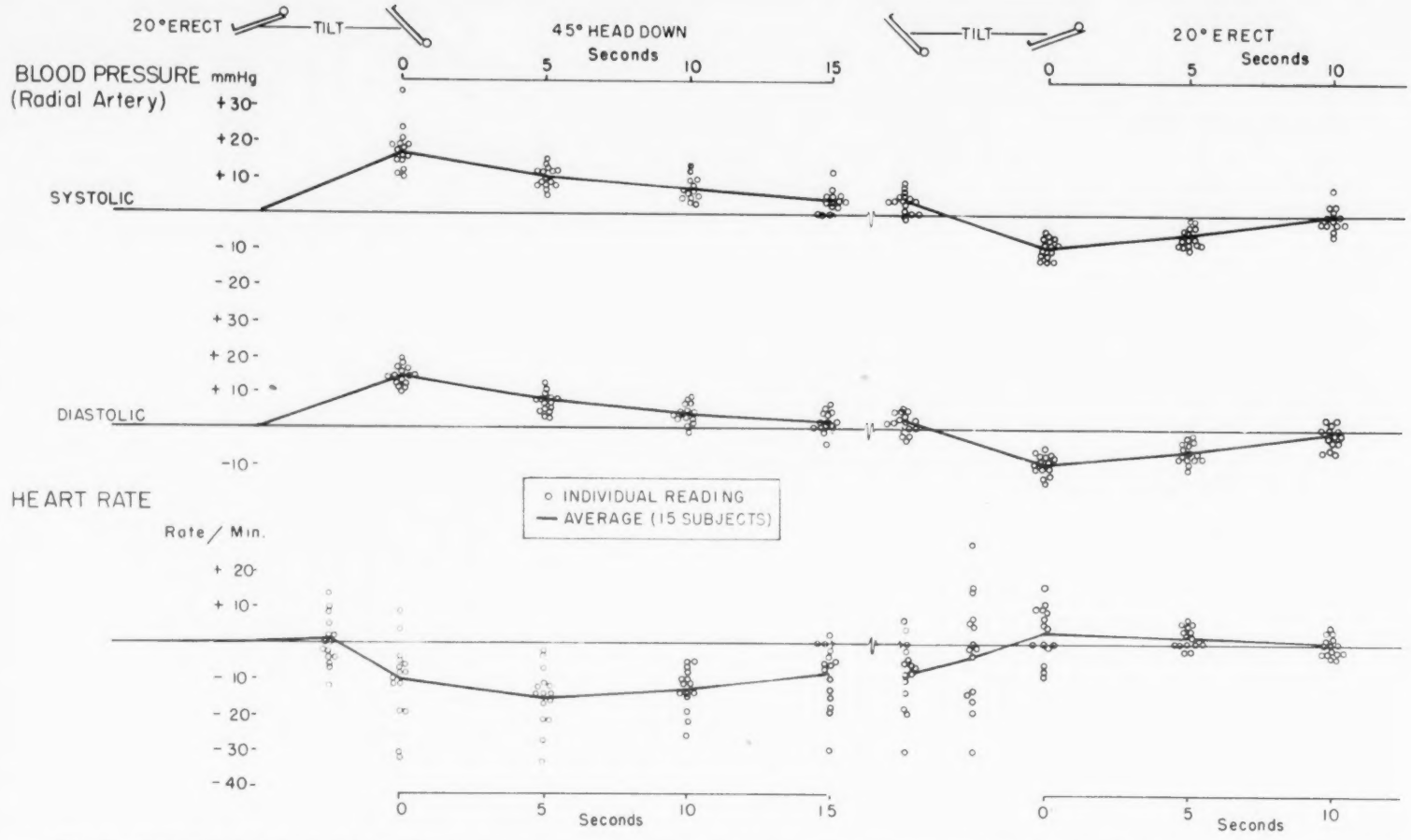

Fig. 2.—Normal heart rate and blood pressure response to tilting. The base lines represent the blood pressure and pulse readings immediately prior to the head-down tilt. The duration of tilting is indicated by the tilt symbols. Changes from the base line values are indicated in millimeters of mercury and beats per minute by the small circles for individual readings and by the solid line for the average readings. Blood pressure was determined by intra-arterial pressure curves, heart rate from the RR interval of the electrocardiogram.

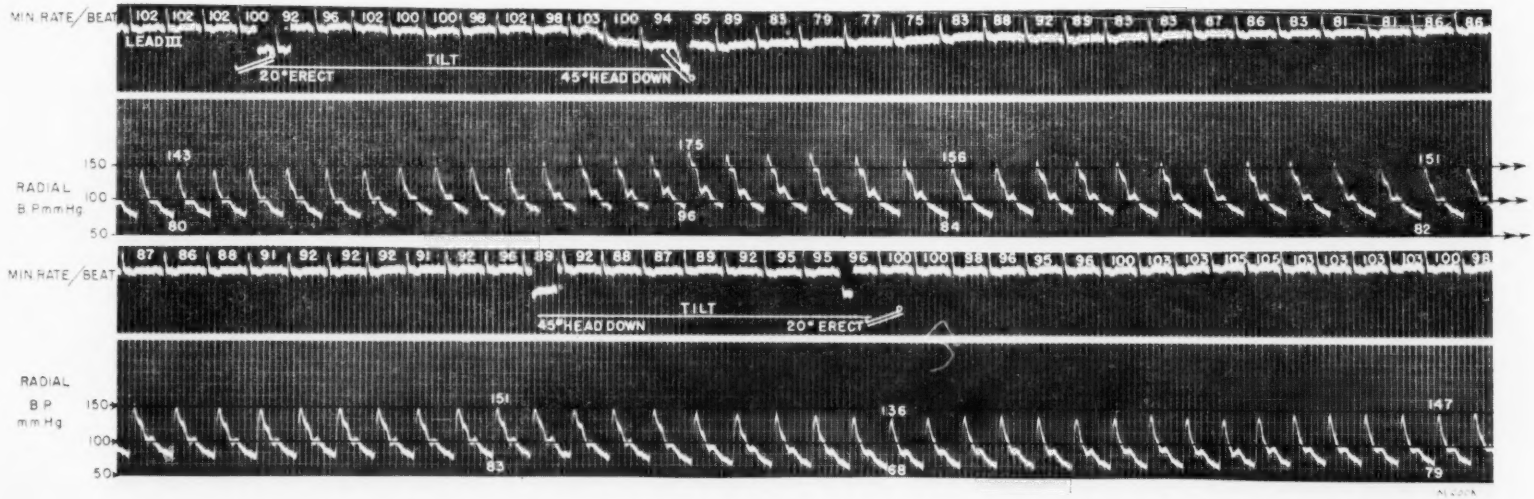

Fig. 3.—Blood pressure and heart rate response to tilting (normal subject F. J., 26-year-old colored man). Normal cardiovascular system. Continuous radial intra-arterial pressure graph and electrocardiogram recorded during and after tilting as indicated. The vertical white lines represent one-tenth second intervals. Blood pressure: During tilting to the head-down position the pressure rose from 143/80 to 175/96. With this position maintained, the blood pressure fell and thirteen seconds later reached a level of 151/82. During the return tilt the blood pressure fell from 151/83 to 136/68. Within ten seconds it returned to 147/79, approximately the initial erect level. Heart rate: In the erect position the rate was 102. In the head-down position and following the elevation of pressure the rate abruptly slowed to 84 per minute for the five-second period immediately following the onset of slowing. The rate then gradually increased, stabilizing within sixteen seconds at a rate of 90 per minute. With the return tilt and following the drop of blood pressure the rate returned to the range of the initial erect level.

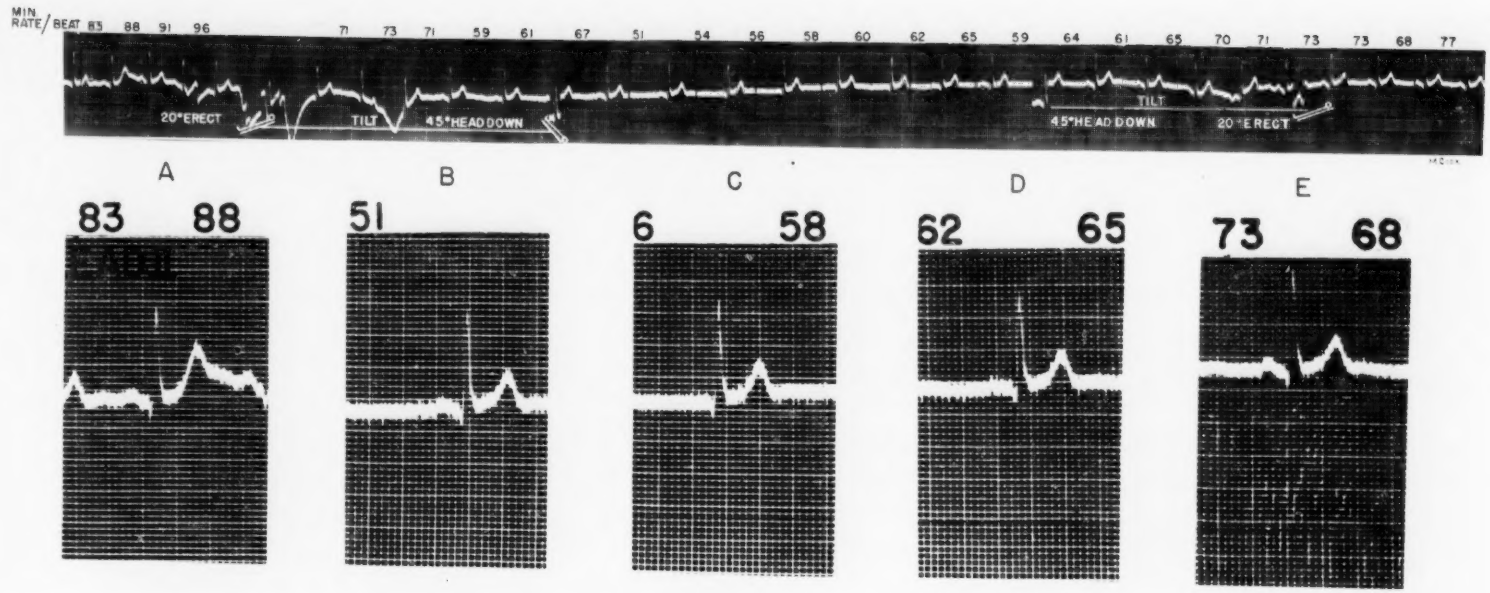

Fig. 4.—Alteration of the P wave during tilting (normal subject). In the erect position (*A*) P waves were present. In the head-down position the P wave gradually decreased in amplitude (*B*) and finally disappeared (*C*). With this position maintained the P wave reappeared and gradually increased in amplitude (*D*). The PR interval preceding the disappearance of the P wave was the same as in the erect position. With the return tilt to the erect position the P wave returned to its initial appearance (*E*).

rate in this position in about half of the subjects. In all instances the rate about ten seconds after the end of the tilt was essentially the same as the original rate (Figs. 2, 3, and 4).

Electrocardiogram.—Our findings on the effect of positional change on the PR interval, the QRS complex, and the T wave of the normal electrocardiogram are in agreement with those reported by other investigators.[13-19] However there are two observations not yet emphasized which we consider worthy of description.

(1) A slowing of the heart rate determined from prolongation of the R-R intervals followed the assumption of the 45 degree head-down position in every normal subject.

(2) In twenty per cent of the normal subjects tilted to the head-down position, the P wave gradually decreased in size and finally disappeared for several beats (Fig. 4). The disappearance of the P wave was not preceded by a shortening of the P-R interval. This change did not invariably occur in the same individual on successive tilting.

DISCUSSION

Blood Pressure.—The changes of the radial and brachial intra-arterial blood pressure recorded in these experiments were presumably a reflection of the blood pressure changes in the large arteries that arise from the aortic arch because the arm was maintained throughout the tilt in a position in which the force of gravity of the column of blood within the arteries of the arm remained constant. Our studies have shown that the blood pressure recorded from these arteries invariably rose during the tilt from a head-up to a head-down position and fell during the reverse tilt. In other experiments we have demonstrated that the blood pressure recorded from the femoral artery always fell during the tilt from the head-up to the head-down position and rose during the return tilt.[20] These findings indicated that the primary alterations of pressure that accompanied the postural changes were due to a shift of the hemostatic force of gravity of the column of blood enclosed by the aorta and its branches and were not secondary to reflex changes. These primary alterations of blood pressure apparently acted as intra-arterial stimuli to the regulatory mechanism that controls blood pressure.

In the subjects with normal cardiovascular systems these primary alterations of radial or brachial pressure were followed by a characteristic pattern of depressor response in the head-down position and of pressor response in the erect position whereby the blood pressure in the region of the aortic arch returned within eight to eighteen seconds to a level that was approximately the same regardless of the position of the body.

The mechanism of blood pressure regulation has been explored extensively in animals. An elevation of arterial blood pressure stimulates the proprioceptors of the carotid sinus region,[21, 22] the left ventricle, the aortic arch, the thoracic aorta,[23, 24] and the abdominal aorta.[25] This induces reflex dilatation of the splanchnic and peripheral arterioles[22, 23] and of the large thyroid vascular

bed,[26] intracranial-extracranial anastomoses,[27] and slowing of the heart rate,[21-23, 28] resulting in a return of the blood pressure to its previous level. A fall of arterial pressure induces a diametrically opposite type of vasomotor response.

The presence of both pressor and depressor regulatory responses in human subjects with normal cardiovascular systems has been demonstrated in this study. However, exact analysis of these responses in man awaits further investigation of the sites of initiation, the mechanisms of regulation, and the physical factors that determine the degree of blood pressure rise that occurs during tilting. That man has vascular reflexes which influence blood pressure has been proved through the results of denervation operations and by the fact that stimulation of the carotid sinus area may produce both bradycardia and fall in blood pressure.[29] Evidence that reflexes are important in the postural control of blood pressure is found in the peripheral dilatation[30, 31] and the bradycardia that occur on assumption of a head-down position.

The function of the regulatory mechanisms that maintain the pressure in the upper aorta at a relatively stable level can be most clearly thought of as a reaction to protect the brain (and probably the heart) against arterial pressure changes that might harm the individual. At one extreme a marked elevation in intracranial arterial pressure might conceivably result in brain damage, and at the other a lowering of cerebral arterial pressure might lead to cerebral anoxia and syncope.

Heart Rate.—In a normal subject an inverse relationship of heart rate to blood pressure has always followed the change of blood pressure produced by tilting. Similar alterations in heart rate have been noted subsequent to the change of blood pressure that occurs during and after the Valsalva experiment[32] and following the occlusion or opening of arteriovenous fistulas.[33]

The relation between the position of the body, the blood pressure, and the heart rate in these experiments suggests that the change of rate is one of the reflex mechanisms that regulates blood pressure. In the head-down position the initial slowing greatly resembled a braking action. In addition the stabilized rate in this position was characteristically slower than the rate in the erect position even though the blood pressure had returned to the erect level. This indicated that this relative bradycardia was one of the means whereby the potential increase of pressure due to the weight of the column of blood (now directed toward the upper arterial regions) was counteracted.

Although it has been known for many years that slowing of the heart may occur in the head-down position, the universal appearance of this reaction in subjects with normal cardiovascular systems has not been appreciated. This is probably due to the fact that the slowing is often of short duration and is detected only through a continuous method of recording.

The disappearance of the P wave for a few beats in a large proportion of normal subjects tilted to the head-down position cannot be explained at present. The fact that the disappearance was preceded by a gradual decrease in amplitude of the P wave and was followed by a gradual increase in amplitude is

evidence that position alone was not the responsible factor. The fact that the changes of the P wave were not associated with a shortening of the P-R interval indicates that the resultant rhythm was not nodal.

In 1897 Hill[3] concluded from his experimental work on the blood pressure and heart rate of animals that the effects of changing the position afford a most delicate test of the vasomotor mechanism. The development of a suitable method of blood pressure recording has made it possible for us to demonstrate that this conclusion is probably valid for human subjects. We feel that this method of study may prove to be of value in obtaining a better understanding of the normal physiology of cardiovascular reflexes and of the influence of disease and drugs on these responses.

SUMMARY AND CONCLUSION

A graphic record of radial or brachial intra-arterial blood pressure during and after tilting at a moderate rate between the 20 degree erect and the 45 degree head-down position was obtained in more than two hundred subjects. In this study careful analysis was made of the response of heart rate and blood pressure to tilting in fifteen healthy young adults in order to establish a standard to evaluate changed reactions due to disease or drugs.

Electrocardiograms were recorded in one hundred normal adults during and following tilting.

An immediate elevation of blood pressure occurred in all subjects studied during the tilt from the 20 degree erect to the 45 degree head-down position. An immediate fall occurred during the return tilt from the 45 degree head-down to the 20 degree erect position.

In the normal subjects these primary alterations of blood pressure were followed by a characteristic pattern of depressor response in the head-down position and of pressor response in the erect position whereby the blood pressure returned in from eight to eighteen seconds to a level that was approximately the same regardless of the position of the body.

The heart rate invariably slowed in the head-down position and increased in the erect position.

In twenty per cent of the normal subjects tilted to and maintained in a head-down position, the P wave gradually decreased in size and finally disappeared for several beats. The disappearance of the P wave was not associated with a change of the P-R interval. This change did not invariably occur in the same individual on successive tiltings.

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DICUMAROL IN EXPERIMENTAL MYOCARDIAL INFARCTION

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THE first report of the influence of anticoagulants on coronary thrombosis was that of Solandt and Best in 1938[1] who used heparin successfully in experimental animals. Since 1945 many articles have appeared in the medical literature[2] describing the use of Dicumarol for patients with acute myocardial infarction. The first patients to receive anticoagulant[2a] therapy were selected because, (1) "They had suffered repeated episodes of multiple thrombi in different areas of the coronary tree, or the original thrombosis had propagated . . . ," or (2) "They had suffered repeated embolic phenomena either pulmonary or to other areas. . . ." It is apparent that the initial indications were based on analogy with the rationale of anticoagulant therapy in other forms of thromboembolic disease. Because of the beneficial effects of Dicumarol, its use was not long restricted to patients with the complications noted; it was given to patients with myocardial infarction as a prophylaxis against such complications. Dicumarol is generally employed in preference to heparin because of the lesser expense and the convenience of the oral route of administration. All who have reported on its use have emphasized the indispensability of accurate prothrombin time determinations to control the dosage.

The incidence of thromboembolic complications in patients with recent myocardial infarction varies in different series of cases. The conflicting data are difficult to analyze and compare since the origin of emboli is seldom determinable with certainty. Hellerstein and Martin[3] found thromboembolic lesions at autopsy in 45 per cent of 160 consecutive cases. Nay and Barnes[4] found thromboembolic complications in 37 per cent of 100 consecutive patients with myocardial infarction. Blumer[5] found 16 per cent in his series of 175 patients; Conner and Holt[6] reported 10 per cent in their series of 287 patients, and Mintz and Katz[7] observed 9.9 per cent in a series of 572 patients with recent myocardial infarction. These data include not only thromboembolic phenomena arising in the heart, but also phlebothrombosis, thrombophlebitis, and so forth. The incidence of mural thrombosis of the endocardium is also difficult to ascertain. In autopsy series, the reported incidence varies from 17 per cent to 83 per cent.[8] It is evident from these reports that thromboembolism is an important complication of myocardial infarction. In all the reports of the results of anticoagulant therapy there has been an apparently significant reduction in the number of thromboembolic complications and in the general mortality rate. There has been as yet no report which includes suitable controls, but such studies are now in progress at a number of centers under the auspices of the American Heart Association. It appears likely that anticoagulants will be used to an increasing extent in the treatment of patients with recent myocardial infarction.

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The chief hazard with anticoagulant therapy is the development of a hemorrhagic state. It is customary to consider any abnormal bleeding tendency, hepatocellular disease, and renal insufficiency as contraindications to the use of Dicumarol. There are no reports in the literature of toxic effects of Dicumarol therapy other than the specific suppression of prothrombin production. Wright[21] mentioned "The possibility that intimal hemorrhage might be a complicating factor (in coronary thrombosis) . . . but no evidence was obtained in this series, either clinically or pathologically, that this was of significance in any case." He also noted that "In no instance was it felt that Dicumarol influenced the rhythm or the rate of the heart directly." It occurred to us that the decreased coagulability of the blood resulting from Dicumarol (or heparin) therapy might affect adversely the course of the myocardial infarct. The early stages of these infarcts are characterized by hyperemia and hemorrhage, which is then followed by necrosis and organization with fibrosis. It seemed possible that the use of anticoagulants could accentuate the hemorrhagic stage of the infarct and thus prolong its resolution. Furthermore, there were no reports in the literature of side effects of Dicumarol which might be advantageous or disadvantageous to the infarcted heart. Such effects might involve facilitation or depression of autonomic reflexes arising in the infarcted myocardium.[9] They might also lead to alterations in conduction leading to changes in the electrocardiogram.

The present study was undertaken to investigate the influence of Dicumarol therapy on the healing of experimental myocardial infarcts. Attention was directed particularly to: (1) the mortality rate after the administration of Dicumarol, (2) the extent and character of the infarct, grossly and microscopically, (3) the evolution of the electrocardiographic changes, and (4) the behavior of the sedimentation rate. Anterior infarcts produced by ligation of the anterior descending branch of the left coronary artery were studied because it has been the experience in our laboratory, as well as elsewhere,[9a] that these have a low immediate mortality—less than 25 per cent of the experimental dogs die within twenty-four hours and virtually none die later of cardiac failure. Mural thrombosis of the endocardium, or other obvious thromboembolic complications, have not been observed in any of the several hundred dogs with experimental myocardial infarction studied in this laboratory since 1938. On this account it was thought that in dogs any alteration in the mortality rate of experimental myocardial infarction in treated animals would be a primary effect of the Dicumarol, rather than a secondary effect due to the prevention of thromboembolic complications. Since uneventful recovery was the usual finding, deviations from the ordinary course should be easy to recognize.

METHODS

Healthy mongrel dogs of either sex were used. Control electrocardiograms were obtained, and blood was drawn for the determination of the prothrombin time, the hematocrit, and the sedimentation rate. Prothrombin time was determined by the method of Quick[10] using a potent rabbit brain thromboplastin. The concentration of the thromboplastin was adjusted so that a value of 6 seconds was obtained with normal dog plasma. This value was assumed to rep-

resent 100 per cent prothrombin time. When longer values for prothrombin time were obtained during Dicumarol therapy they were expressed simply as per cent of the normal value of 6 seconds. This relationship is shown in Table I. In some instances prothrombin time was determined with 12.5 per cent plasma; the average normal value was 16.5 seconds, with a range of 13 to 23 seconds. The sedimentation rate was measured in a Wintrobe tube, with one reading made at one hour. The hematocrit was determined in the same tube by centrifuging at high speed to constant volume.

TABLE I. RELATIONSHIP BETWEEN PROTHROMBIN TIME IN SECONDS AND PER CENT PROTHROMBIN TIME*

PROTHROMBIN TIME (SEC.)	PER CENT PROTHROMBIN TIME
6	100%
7.5	80%
9	60%
12	50%
15	40%
18	30%
21	20%

*It is to be noted that per cent prothrombin time represents: $\frac{\text{Normal control prothrombin time}}{\text{observed prothrombin time}} \times 100$; it does not refer to the dilution of normal plasma which would give the same number of seconds clotting time.

One-half hour before operation each dog was given a subcutaneous injection of 1.0 mg. per kilogram morphine sulfate, and 0.03 mg. per kilogram atropine sulfate. General anesthesia was produced by the slow intravenous injection of solution of sodium pentobarbital, approximately 30 mg. per kilogram. A tracheal catheter was inserted and positive pressure ventilation with oxygen was obtained with a face mask and a water seal. With aseptic precautions the thorax and the pericardial sac were opened from the left side. A ligature was passed around the anterior descending branch of the left coronary artery between its origin and its first major branch. A satisfactory infarct was observed in every instance. Several cubic centimeters of penicillin solution (10,000 units per cubic centimeter) were placed in the pericardial and pleural spaces, the lung was reinflated, and the thorax was closed in layers.

When they regained consciousness the dogs that were to receive Dicumarol were given a first dose of 50 or 100 mg. in a bolus of ground meat. The Dicumarol treated group were given the drug subsequently according to the following schedule:

Prothrombin time less than 15 seconds; or more than 40 per cent prothrombin time: 50 mg. Dicumarol.

Prothrombin time from 15 to 25 seconds; or between 40 and 24 per cent prothrombin time: 25 mg. Dicumarol.

Prothrombin time more than 25 seconds; or less than 24 per cent prothrombin time: no Dicumarol.

Serial determinations of the prothrombin time, the hematocrit, and the sedimentation rate, and serial electrocardiograms were made on all dogs. Typical sample protocols are shown in Tables IV and V.

The animals were sacrificed at intervals of five to twenty-two days after the ligation of the coronary artery. The time of death and the distribution of the groups are shown in Table II. The hearts were removed, cut open, and fixed in Kaiserling I solution. Subsequently the color of the tissues was developed in Kaiserling III solution and kodachrome photographs were obtained. Suitable blocks of tissue were removed for histologic examination.

RESULTS

Seven of the thirty-two dogs died within twenty-four hours after the production of coronary occlusion, giving an immediate mortality of 21.8 per cent. (See Table II.) There were no deaths attributable to cardiac failure after the

TABLE II. EXPERIMENTAL DATA

Died within 24 hours after coronary occlusion (21.8%)		7
Control group		
Sacrificed during first week	2	
Sacrificed during second week	4	
Sacrificed during third week	4	
Total		10
Dicumarol treated group		
Sacrificed during first week	2	
Sacrificed during second week	3	
Sacrificed during third week	8	
Died: hemorrhage/infection on eighth and eleventh day respectively	2	
Total		15
Total experimental coronary occlusions		32

first day. Five of the dogs treated with Dicumarol developed complications due to the abnormal hemorrhagic tendency that resulted. Two of these died, apparently from anemia, and the other three became severely anemic. The details of the findings in these animals are shown in Table III. All of the animals but

TABLE III. HEMORRHAGIC COMPLICATIONS OF DICUMAROL THERAPY

DOG	DIED (DAY)	SACRIFICED (DAY)	LONGEST PROTHROMBIN TIME (SEC.)	LOWEST HEMATOCRIT (%)	DICUMAROL (AV. DAILY DOSE, MG.)	AUTOPSY FINDINGS
8		11	50 (12%)	16	40	Hemothorax
9	13		37 (16%)	15	20	Hemothorax; gastrointestinal hemorrhage
11		16	30 (20%)	15	25	
14	8		65 (9%)	32*	25	Ulcerative colitis; gastrointestinal hemorrhage; hemothorax
17		21	65 (9%)	10	16	Gastrointestinal hemorrhage

*This value may represent relative hemoconcentration, resulting from the severe diarrhea.

one developed typical myocardial infarcts. There was no mural thrombosis in any animal, and there was no evidence that any thromboembolic phenomena occurred. Typical protocols for a control dog, Dog 16 (Fig. 1), and for a dog treated with Dicumarol, Dog 21, are shown in Tables IV and V respectively.

TABLE IV. PROTOCOL: DOG 16, BROWN, SHORT-HAIRED MALE, WEIGHT, 11.5 KILOGRAMS

DAY	ECG	SEDIMENTATION RATE	HEMATOCRIT	PROTHROMBIN TIME		DICUMAROL (MG.)
				QUICK	12.5 PER CENT	
−1	067	13	42	6	14	--
0						
1	072	--	46	6	20	--
3	078	15	29	5.5	9.5	--
6	086	40	40	5.5	8.5	--
7	--	43	43	5.5	9	--
8	090	--	--	--	--	--
10	096	24	42	6	13	--
12	102	--	--	--	--	--
14	108	24	35	6	11	--
17	113	--	--	--	--	--
Sacrificed						

Autopsy: There was no evidence of wound infection or of pleural effusion or hemothorax. There were two ligatures imbedded in considerable pericardial fibrinous exudate: one on the artery and one on the vein which was accidentally divided during the surgical procedure. There was a large and very clearly demarcated infarct of the apical region.

TABLE V. PROTOCOL: DOG 21, BLACK AND WHITE MALE SHEPHERD, WEIGHT, 13.5 KILOGRAMS

DAY	ECG	SEDIMENTATION RATE	HEMATOCRIT	PROTHROMBIN TIME		DICUMAROL (MG.)
				QUICK	PER CENT	
−1	106	2	44	6	100	--
0						
1	112	8	44	6	100	50
2	--	--	--	--	--	50
4	117	4	42	12.5	48	50
6	121	14	36	22	27	25
7	--	--	--	--	--	25
8	127	22	34	45	13	--
10	--	--	--	--	--	25
11	132	22	37	12	50	50
13	137	24	35	20	30	25
14	--	--	--	--	--	25
15	139	20	38	19	32	25
17	--	--	--	--	--	25
18	143	18	40	12	50	50
20	145	11	45	16	38	25
21	149	--	--	Sacrificed		

Autopsy: The wound was well healed. There were adhesions between lung and chest wall. There was no effusion. There was a slight pericardial effusion, the fluid of which was serosanguineous in character. There was a small linear area of fibrosis overlying the region of the interventricular septum and some apparent thinning of the apex of the left ventricle. This was a well-healed infarct.

The control electrocardiograms were analyzed with respect to the features consistently affected by myocardial infarction. The findings for twenty of the dogs that survived are shown in Table VI. After occlusion of the anterior descending branch of the left coronary artery, changes resembling the human Q_1T_1 pattern were observed in fifteen of twenty-one animals whose serial electrocardiograms were satisfactory for analysis. An example of the characteristic changes is shown in Fig. 1. The typical features occurred in 87 per cent of the control animals and in 61 per cent of those receiving Dicumarol. See Table VII, A. It is doubtful that this small difference is significant. The occurrence of changes in Q_1, T_1, T_2, T_3, and ST segments for the entire group (controls and treated) is summarized in Table VIII. The rate of evolution of the Q_1T_1 pattern in each

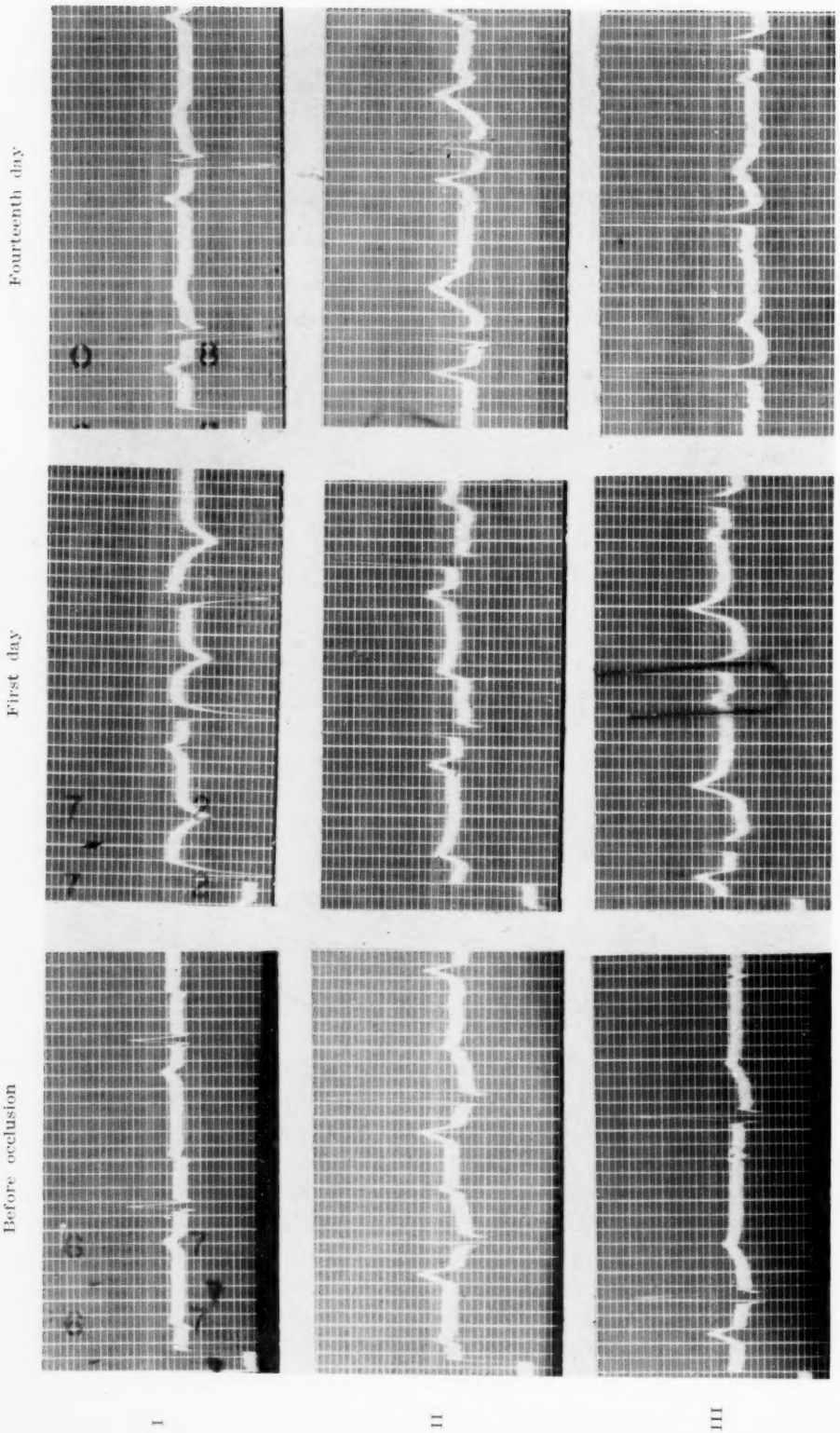

Fig. 1.—Dog 16, control, typical ECG—Q T_1.

TABLE VI. ELECTROCARDIOGRAM FINDINGS IN TWENTY CONTROL DOGS

Q_1		T_2	
Absent	4	Upright	10
Present	4	Diphasic	3
Deep	12	Isoelectric	0
		Inverted	7
T_1		T_3	
Upright	11	Upright	9
Diphasic	1	Diphasic	1
Isoelectric	1	Isoelectric	1
Inverted	7	Inverted	9

ST Segments, Any Lead	
No deviation	17
Depressed, elevated	3

TABLE VII. ANALYSIS OF SERIAL ELECTROCARDIOGRAMS

A. OCCURRENCE OF TYPICAL Q_1 T_1 PATTERN IN:	
Controls	7 out of 8
Dicumarol treated	8 out of 13
Total	15 out of 21
B. OCCURRENCE OF ARRHYTHMIAS	
Premature ventricular contractions in:	
Controls	6 out of 8
Dicumarol treated	13 out of 13
Premature nodal contractions and bundle branch block in:	
Controls	0 out of 8
Dicumarol treated	4 out of 13

TABLE VIII. EFFECT OF ANTERIOR INFARCTION ON Q_1T_1 PATTERN IN SERIAL ELECTROCARDIOGRAMS, TWENTY-ONE SATISFACTORY RECORDS

Q_1		T_1	
No change	7	No change	1
Control to deep	13	Upright to inverted	4
Deep to absent	1	Upright to inverted to upright	9
		Inverted to upright	7
T_2		T_3	
No change	10	No change	14
Upright to inverted	7	Upright to inverted	4
Upright to inverted to upright	2	Upright to inverted to upright	1
Inverted to upright	2	Inverted to upright	2

ST Segment	
Reciprocal displacement, Lead I and III	11
No reciprocal displacement	10

group is summarized in Table IX. There was no difference in the rate of evolution of the changes in Q_1. In the animals that received Dicumarol, inverted T_1 returned to normal on an average of one day later than in the control dogs. The significance of this difference also is doubtful. Arrhythmias (see Table VII, B) were found more frequently in the electrocardiograms of the dogs that received Dicumarol. However it is our experience that when electrocardiograms are made daily in dogs, very few fail to show some arrhythmia after coronary occlusion. In the present experiment, tracings were not always made daily

during the first week when the transitory arrythmias occurred. Bundle branch block occurred twice in the treated group, but this is not infrequent as a transient occurrence in dogs with myocardial infarction.

TABLE IX. EVOLUTION OF Q_1 T_1 PATTERN IN SEVENTEEN ANIMALS SACRIFICED IN SECOND AND THIRD WEEK AFTER CORONARY OCCLUSION

	Q_1
Controls (6)	
	Five developed deep Q_1.
	One had no significant change in Q_1.
	One returned to normal on twenty-first day.
	Four were sacrificed before change occurred.
Dicumarol treated (11)	
	Six developed deep Q_1.
	Five had no significant change in Q_1.
	Five were sacrificed before change occurred.
	One returned to normal on fourteenth day.
	T_1
Controls (6)	
	Five developed inverted T_1.
	Five returned to normal by eighth day.
	One had no significant change in T_1.
Dicumarol treated (11)	
	Five developed inverted T_1.
	Four became normal by ninth day.
	One died on eighth day, T_1 inverted.
	Six had no significant change in T_1.

With one exception all the hearts removed at autopsy were found to have infarcts of the apical and anterior septal regions, with myocardial, subepicardial, and subendocardial hemorrhage. The sole exception, Dog 19, a control, had no visible infarct. The ligature was in place and the coronary artery was occluded. Large anastomotic branches were easily seen connecting the occluded branch with the circumflex branch of the left coronary artery. There did not appear to be any significant, consistent difference in the size of the infarcts or the amount of hemorrhagic infiltration in the two groups of animals. This judgment is based on experience with more than 100 dogs' hearts with anterior infarction. Reproductions of kodachrome photographs of the control and the treated hearts sacrificed at similar times are shown in Figs. 2, 3, and 4. Pleural and pericardial adhesions occurred with equal frequency in each group of dogs. It is worthy of comment that the extent of these adhesions was much less than in our previous experience. The difference is most certainly due to the action of penicillin, applied topically, in preventing infection. Microscopic examination of representative sections was performed by Dr. William B. Wartman.* It was his opinion that there was no obvious difference between the healing infarcts in the treated animals and the controls.

Using the Quick method for prothrombin time and following the schedule of dosage of Dicumarol noted previously, it was possible to regulate the clotting activity of the blood in a satisfactory manner. Excessive dosage and the appearance of abnormally high values for prothrombin time occurred over holidays and week-ends when the usual daily dose was continued without laboratory control.

*Professor of Pathology, Northwestern Medical School.

Fig. 2.—Right: Dog 3, control, sacrificed on the fifth day. Left: Dog 4, sacrificed on tht fifth day, received a total of 250 mg. Dicumarol; maximum value for prothrombin time was 15 seconds. There is very little difference in the appearance of these two hearts.

Fig. 3.—Right: Dog 15, sacrificed on the seventeenth day, received a total of 425 mg. Dicumarol; maximum value for prothrombin time was 33 seconds. Left: Dog 16, control, sacrificed on the seventeenth day. There is no difference in the appearance of these two hearts.

In every instance in which abnormal bleeding occurred, the prothrombin time was longer than 25 seconds. In the control group the prothrombin time was shorter than the lower limit of normal during the first week after occlusion in four of the seven animals with satisfactory serial tests. (See Table IV.) Increased clotting activity of these dogs was apparent only when 12.5 per cent

Fig. 4.—Right: Dog 17, sacrificed on the twenty-first day, received a total of 350 mg. Dicumarol; the maximum value for prothrombin time was 65 seconds. Left: Dog 11, sacrificed on the sixteenth day, received a total of 325 mg. Dicumarol; the maximum value for prothrombin time was 30 seconds.

plasma was used for the determination. The variations in the sedimentation rate were not consistent and were of no importance in evaluating the course of experimental myocardial infarction. The hematocrit was a useful index of the extent of such anemia as developed.

SUMMARY

Myocardial infarction was produced in twenty-five dogs by ligation of the anterior descending branch of the left coronary artery. Fifteen of the animals were given Dicumarol in amounts equivalent to those used in the management of patients with recent myocardial infarction. There was no evidence that the altered coagulability of the blood affected the extent or the healing of the infarcts. Serial electrocardiograms did not show any consistent significant difference between the treated animals and the controls.

CONCLUSION

The use of the anticoagulant Dicumarol does not have any demonstrable deleterious influence on the healing of experimental myocardial infarction in dogs.

The authors wish to express their gratitude to Miss Ruth A. Trump, B.Sc., for her painstaking laboratory work.

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THE EFFECTS OF DICUMAROL ON THE ELECTROCARDIOGRAM

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THROMBOEMBOLIC complications long have been recognized as a major hazard in the course of acute myocardial infarction.[1-3] Increased blood clotting tendency following myocardial infarction has been demonstrated repeatedly.[4, 5] Anticoagulant therapy with Dicumarol has been invoked accordingly by many clinicians as a prophylactic measure to neutralize the increased propensity to thromboembolism following myocardial infarction. In the past several years sufficient experience with this drug has accumulated to show substantial (in some reports even remarkable) reduction in thromboembolic catastrophes.[5-8] Our own favorable experience thus far has been sufficiently persuasive to justify addition of controlled Dicumarol therapy to the routine regimen for every patient with acute myocardial infarction.

There are scattered references in the literature to the relative harmlessness of Dicumarol so far as the heart is concerned; Blumgart and co-workers showed that in dogs whose coronary arteries had been ligated Dicumarol had no adverse effects on the evolution of the infarction per se.[5, 7] In a field where electrocardiographic developments are depended on for evaluation of myocardial change, it becomes important to determine whether any of the electrocardiographic changes are due to the drug taken. The purpose of this study was to establish whether Dicumarol per se has any effects on the electrocardiogram, and, if so, what these effects are.

METHODS

Dicumarol was given to forty-eight subjects. Of these, twelve were normal, with no discoverable evidence of cardiovascular disease and with repeatedly normal electrocardiograms (Group 1). Sixteen were patients with varying types of cardiovascular disease and a variety of abnormal electrocardiographic patterns which remained stable during preliminary observation (Group 2). Daily electrocardiograms were taken at each step of the progressive fall in the prothrombin level until prothrombin activity between 15 and 25 per cent of normal was attained. In several instances Dicumarol dosage was deliberately increased until prothrombin concentration fell to less than 10 per cent of normal so as to observe the effects of such dosage on the electrocardiogram. Inasmuch as in our cases of acute myocardial infarction anticoagulant therapy is continued until the patient becomes semiambulatory, the effect of adequate Dicumarol medication continued for at least four weeks was observed in five of the normal subjects and in four of the subjects with cardiovascular disease (Group 1 and Group 2 respectively).

In addition we studied the serial electrocardiograms in fourteen of our patients with acute infarction who were on a Dicumarol regimen (Group 3) for evidence of possible Dicumarol effects, both during the period of Dicumarol treatment and following withdrawal of the drug.

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A separate group of six digitalized patients (Group 4) whose electrocardiograms had remained stable under prolonged observation were also treated with Dicumarol in the manner described to observe the possible modifying influence of the drug on the several degrees of digitalis effect.

In all subjects a preliminary study of renal function was done and a pretreatment prothrombin level was obtained. In the course of Dicumarol administration the drug effect was closely controlled with daily prothrombin level determinations, and toxicity was watched for by daily study for microscopic hematuria, examination of the skin for hemorrhagic extravasations, and inquiry about bleeding from gums, nose, and gastrointestinal tract.

Aside from the maintenance dose of digitalis received by the six patients of Group 4, the only medication given was an occasional dose of aspirin, and, in Group 3, opiates and papaverine during the early stages of acute myocardial infarction.

Leads I, II, III, CF_2, CF_4, and CF_5 were taken in all instances. Electrocardiograms were analyzed with respect to rhythm, auriculoventricular and intraventricular conduction, Q-T duration, RS-T level, and amplitude of auricular and ventricular components.

The Quick method was used to determine prothrombin activity. Each batch of thromboplastic substance was calibrated against a number of normal sera in several dilutions, to minimize the errors of interpolation. Results were expressed as percentage of normal prothrombin activity.

RESULTS

In Groups 1 and 2 all electrocardiograms taken at successively lower prothrombin levels were substantially the same. An occasional tracing showed minimal changes in amplitude of T_1, and more rarely in amplitude of P, commensurate with the associated changes in rate. In the precordial leads, minor changes in R/S ratio were seen, attributable to slight shifting of the exploring electrode during successive tracings.

In those subjects maintained for several weeks on adequate doses of Dicumarol no appreciable change appeared in the electrocardiograms as a result of the prolonged medication.

Subjects given maximal doses of Dicumarol, with depression of prothrombin to less than 10 per cent of normal, showed no electrocardiographic effects of their hypoprothrombinemia.

The serial electrocardiograms of patients with acute myocardial infarction (Group 3) showed the expected progressive changes of healing infarction. The rate of progress did not differ materially from that observed in patients not receiving Dicumarol. Withdrawal of the drug during convalescence did not cause significant electrocardiographic change in any of these cases. The residual electrocardiographic abnormalities in this small series fell within the expected range of late postinfarction patterns.

All of the group of patients whose electrocardiograms showed abnormalities attributable to digitalis (Group 4) retained the respective patterns unchanged by effective doses of Dicumarol, and the electrocardiograms remained stable after withdrawal of the Dicumarol. In one of these patients occasional ventricular extrasystoles appeared in the course of the study and disappeared again when digitalis was withheld for two days. Dosage of Dicumarol was not changed during this interval and the electrocardiogram was not otherwise affected.

Questionable toxic effects were observed in only one of the subjects. On a morning when the prothrombin concentration was 15 per cent of normal, the

patient reported coughing up (or possibly vomiting) a small quantity of blood-streaked material. There was no associated evidence of bleeding in the skin, mucous membranes, or urine, and no change in blood pressure, heart rate, or red cell count. The electrocardiogram that day did not differ from previous and succeeding tracings. The patient was given 20 mg. of synkayvite intravenously and Dicumarol was withheld. There were no further adverse manifestations.

SUMMARY

The addition of Dicumarol to the therapeutic regimen of patients with acute myocardial infarction suggested the investigation of the effects of the drug on the electrocardiogram.

The subjects of this study, forty-eight patients with and without heart disease and with a wide variety of electrocardiographic patterns, were maintained for a period on adequate doses of Dicumarol. Serial electrocardiograms taken during this period were analyzed.

No significant electrocardiographic deviation attributable to Dicumarol was observed in any of the subjects.

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THE LIFE SPAN OF THE SICKLE CELL AND THE PATHOGENESIS OF SICKLE CELL ANEMIA

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THE mechanism responsible for the development of sickle cell anemia is not yet understood. Only relatively few Negroes who harbor the sickle cell trait are afflicted with this hemolytic syndrome. Recent in vitro studies of the sickling phenomenon in our laboratory[1] failed to demonstrate any fundamental difference between trait and anemia cells. When susceptible erythrocytes are suspended in a culture of *Bacillus subtilis*, either sickling or oat cell formation occurs regularly after from five to fifteen minutes.[1] No distinct correlation could be detected between the number of sickle cells in the preparation or the rapidity and degree of the sickling process and the presence of either trait or anemia. Therefore, it seemed conceivable that an additional qualitatively different factor might be operating in the production of the anemia.

As has been pointed out in a previous paper,[2] the various hemolytic syndromes may quite generally be classified in two main groups. The first comprises all the disorders in which red cells are damaged by an extracorpuscular mechanism (for example, malaria). In the disorders belonging to the second group, premature disintegration of the erythrocytes is apparently caused by a primary abnormality of the stroma which may manifest itself morphologically (for example, familial hemolytic jaundice). This classification is based on the so-called cross determination of the survival time of red cells.[2] When normal erythrocytes transfused into a recipient with a hemolytic syndrome survive normally, whereas the recipient's own red cells transfused into a normal person have a considerably shortened life span, an intracorpuscular anomaly may be suspected. Contrariwise, when normal red cells transfused into the patient with the hemolytic syndrome are as rapidly destroyed as the patient's own cells, the presence of an extracorpuscular mechanism may be assumed.

This paper deals with cross determinations of the survival time of sickle cells. Trait cells were transfused into patients with sickle cell anemia and anemia cells into healthy recipients displaying the sickle cell trait.

MATERIAL AND METHODS

The diagnosis of sickle cell anemia in patients used in this study was based on the presence of a marked or moderate anemia, increase of the reticulocyte count, and elevation of the serum bilirubin, besides a compatible clinical pic-

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ture and a positive sickle cell test. Almost all individuals considered to represent instances of the sickle cell trait had entirely normal hematologic findings except for the positive sickle cell test. Each patient had at least two complete hematologic examinations before being chosen either as donor or as recipient.

Hematologic Determinations.—Five cubic centimeters of blood were obtained by venipuncture and transferred to a vial containing 4 mg. potassium oxalate and 6 mg. ammonium oxalate. From this oxalated blood the following determinations were regularly performed.

Hemoglobin: Photoelectric method of Sheard and Sanford[3] (Evelyn colorimeter); 100 per cent equals 15.6 Gm. per 100 c.c. of blood.

Red Cell and White Cell Counts: United States Bureau of Standards certified pipettes and hemocytometer used. Automatic shaker.

Hematocrit: Centrifugation in Wintrobe type of hematocrit tubes for thirty minutes at 3,500 revolutions per minute.

Bilirubin in the Plasma: Method of Malloy and Evelyn.[4]

Sickle Cell Test: The rapid method of Singer and Robin[1]

Furthermore, smears were prepared from capillary blood on cover slips and stained with Wright's stain. The reticulocyte count was obtained by the dry method.

Methods Used for the Determination of the Survival Time.—Determinations of the survival time were done with the method of differential agglutination (Ashby technique[5]). Either O cells were transfused into recipients belonging to group A or B, or, if donor and recipient were of the same group, the donor's cells containing the N agglutinogen were introduced into an M or MN recipient. Anti-M serum was then employed for agglutinating out the recipient's own cells in the follow-up studies. Anti-N serum was used only for typing and not for any quantitative evaluation on account of its known unreliability for this purpose.[6]

Typing for the Landsteiner blood groups was performed with potent sera prepared by the Serum Center.* Testing for the M and N factors was done with sera supplied by the Certified Blood Donor Center.† In all patients the Rh factor was determined by means of a human anti-Rh serum of high titer.

Blood for transfusions was obtained by venipuncture and was transferred into a flask containing 50 c.c. of a modified a.c.d. mixture. Then the plasma was removed and replaced by a sufficient amount of a dextrose saline solution.‡

An adequate replacement transfusion was given to all anemic donors immediately after the bloodletting, thus more than compensating for the clinically undesirable loss of erythrocytes.

For the follow-up study of the transfused cells, the method of Young, Platzer, and Rafferty[6] was found to be very satisfactory. Each recipient had at least two determinations of the nonagglutinable cells. Since very potent sera were used, the number of nonagglutinable cells never exceeded 50,000 per cubic millimeter.

*Michael Reese Research Foundation.

†Jamaica, N. Y.

‡We are indebted to Dr. S. Levinson and Dr. A. Wolf of the Serum Center, Michael Reese Research Foundation, for their invaluable assistance in the processing of the erythrocytes.

Only young children (1 to 6 years old) were selected as recipients. Because of their small circulating blood volume, a correspondingly small transfusion was found sufficient to result in a satisfactorily high addition of the donor's cells per cubic millimeter. Packed cells derived from 200 to 500 c.c. of whole blood were introduced, yielding an initial increase of 540,000 to 2 million cells per cubic millimeter in the recipient's total erythrocyte level. These initial values were established twenty-four hours after transfusion in order to avoid any significant inaccuracy due to an augmentation of the circulating fluid volume,[7] although it is realized that some of the transfused cells already may have been eliminated within the first twenty-four hours. After determining the starting points, follow-up studies were done twice weekly during the first month and from then on every week.

RESULTS

Transfusion of Trait Cells Into Patients With Sickle Cell Anemia.—Experiments of this type were performed in three instances. The hematologic data of the respective donors and recipients are compiled in Table I. Fig. 1

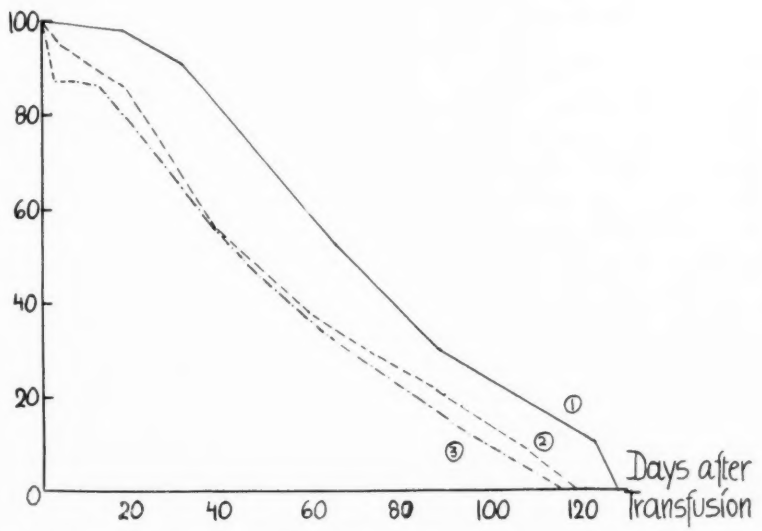

Fig. 1.—Transfusion of sickle cell trait cells into patients with sickle cell anemia, expressed in per cent of surviving donor's cells.

shows the curves illustrating the disappearance of the donor's cells. The percentage of the surviving cells is plotted against the number of days following transfusion. In all three instances the survival time of the trait cells was normal, with an average of 120 days. Thus trait cells, when transfused into patients with sickle cell anemia, have a normal survival time.

Fig. 2 shows the various red cell counts of one of the transfused recipients; these values represent the sum total of two populations of erythrocytes, that is, the recipient's own cells and the donor's cells. As can be seen from the illustrations, the patient's own cells were first hemolyzed much more rapidly than the transfused trait cells. After about thirty days there was an increase of the

TABLE I. TRANSFUSION OF TRAIT CELLS TO PATIENTS

EXPERIMENT	DONOR AGE (YR.)	SEX	HB. GM. %	HB. %	R.B.C. (MILLION)	W.B.C. (1,000)	RETIC. (%)	BILIRUBIN (MG. %)	TYPING FORMULA	AMOUNT OF BLOOD USED FOR TRANSFUSION (C.C.)
1	50	F	12.1	78	4.90	5.9	0.8	0.4	A Rh+ N	500
2	30	F	12.7	82	4.28	5.1	0.2	0.56	O Rh+	400
3	28	F	13.0	84	5.08	12.2	1.0	0.4	O Rh+	400

patient's own cells. When the transfused cells had completely disappeared, approximately the same degree of anemia was present as existed before the transfusion. Similar results were obtained from an analysis of the total counts of the two other patients in this group.

Transfusion of Sickle Cell Anemia Cells Into Recipients With Sickle Cell Trait.—Red cells from four patients with sickle cell anemia were transfused into three patients with sickle cell trait. In one experiment the red cells from two anemic patients both having blood group O were mixed and introduced into a single recipient, thus creating three different populations of red cells in this recipient. The hematologic data of the individuals used in this second group of studies may be found in Table II. Recipient 1 was a patient with iron deficiency anemia harboring the sickle cell trait. This patient had a hypochromic microcytic anemia with absence of target cells and showed a typical reticulocyte response with elevation of hemoglobin and erythrocytes after administration of iron.

TABLE II. TRANSFUSION OF SICKLE CELLS TO PATIENTS WITH SICKLE CELL ANEMIA

EXPERIMENT	DONOR AGE (YR.)	SEX	HB. GM.	HB. %	R.B.C. (MILLION)	W.B.C. (1,000)	RETIC. (%)	BILIRUBIN (MG. %)	TYPING FORMULA	AMOUNT OF BLOOD USED FOR TRANSFUSION (C.C.)	REMARKS
1	8	M	7.9	51	2.92	5.4	11.5	1.6	O Rh+	220	Numerous target cells
2	5	M	7.8	50	2.39	22.4	8.0	1.9	B Rh+ N	2200	Numerous target cells
3a	12	M	7.5	48	2.48	18.3	13.4	2.4	O Rh+	200	Numerous target cells
3b	6	F	9.7	62	3.65	14.9	4.4	0.7	O Rh+	200	Some target cells

TO PATIENTS WITH SICKLE CELL ANEMIA

RECIPIENT									RESULTS	
SEX	HB. GM.	HB. %	R.B.C. (MILLION)	W.B.C. (1,000)	RETIC. (%)	BILIRUBIN (MG. %)	TYPING FORMULA	REMARKS	INCREASE OF INITIAL R.B.C. IN RECIPIENT AFTER TRANSFUSION (MILLION)	SURVIVAL TIME (DAYS)
F	6.2	40	2.12	19.7	16.0	2.5	A Rh+ M	Numerous target cells	2.16	127
M	9.7	62	4.03	9.7	5.0	1.2	A Rh+	Numerous target cells	1.54	118
F	7.9	51	3.33	16.0	16.6	2.4	B Rh+	Numerous target cells 15 Nucleated R.B.C. per 100 W.B.C.	2.01	114
									Average	120

In all instances there was a definite shortening of the survival time* of the anemia cells (Fig. 3). In the first two experiments the anemia cells were no longer demonstrable after thirty and thirteen days, respectively. Of special interest is the profile of Curve 3. At first, 46 per cent of the introduced cells disappeared rapidly within fourteen days; then there was a lag of ten days, which was followed by a further but conspicuously slower decrease of the number of transfused cells. All anemia cells had disappeared after fifty-eight days. Although no proof can be offered, one may interpret this stepped line of decay as being caused by an initial very rapid elimination of the red cells of Donor 3a who had a very active hemolytic process. Then later the remainder of the cells, mostly originating from the second donor (Donor 3b) with a much milder hemolytic syndrome, disappeared more slowly. However, the number of cases

*While these experiments were in progress Callender and Nickel[8] read a preliminary paper at the Twentieth Annual Meeting of the Central Society for Clinical Research on survival time determinations of sickle cell anemia cells in normal recipients. Their results concur with ours. Since their studies also were not completed at that time, their final results and interpretations cannot be taken into consideration here.

EMIA CELLS INTO SICKLE CELL TRAIT CARRIERS

RECIPIENT									RESULTS	
SEX	HB. GM.	HB. %	R.B.C. (MILLION)	W.B.C. (1,000)	RETIC. (%)	BILIRUBIN (MG. %)	TYPING FORMULA	REMARKS	INCREASE OF INITIAL R.B.C. IN RECIPIENT AFTER TRANSFUSION	SURVIVAL TIME (DAYS)
F	8.1	52	5.48	10.5	4.0	0.4	B Rh+	Iron deficiency anemia with sickle cell trait	741.000	30
M	13.8	89	4.86	9.1	0.4	0.5	B Rh+ M		545.000	13
M	12.1	78	4.73	7.5	0.4	0.3	B Rh+		1130.000	58
									Average	34

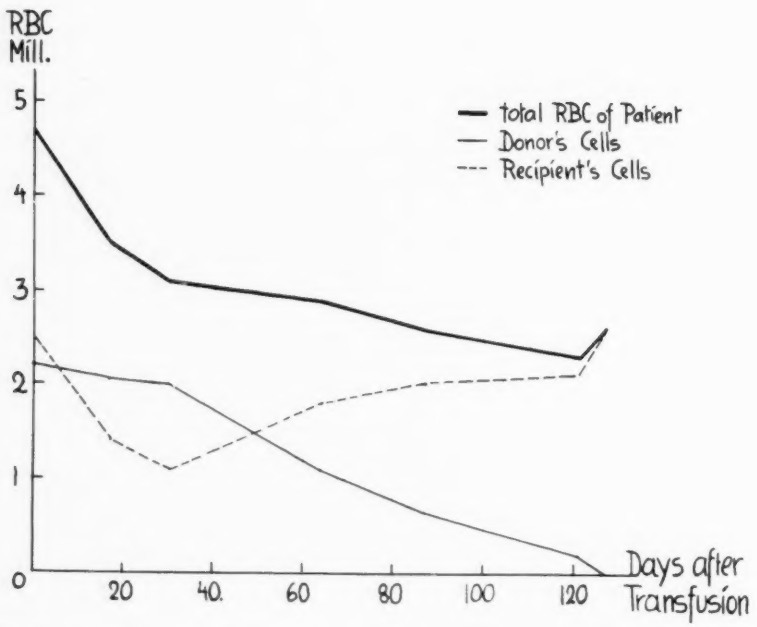

Fig. 2.—Transfusion of sickle cell trait cells into patient with sickle cell anemia.

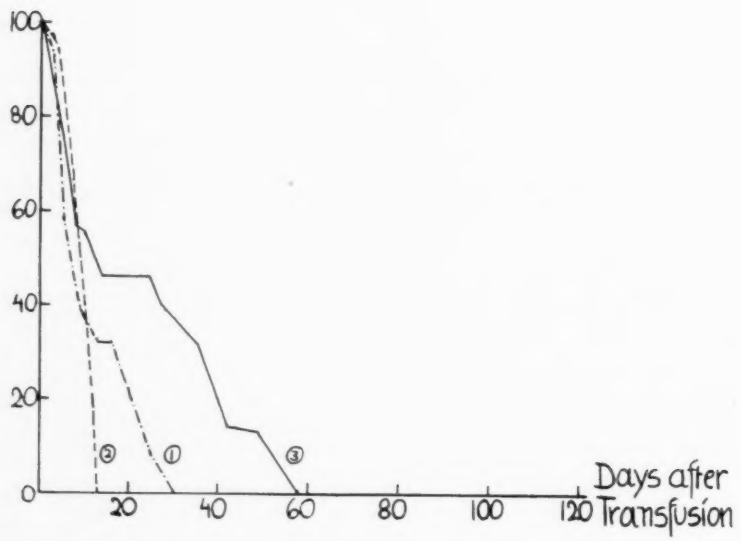

Fig. 3.—Transfusion of sickle cell anemia cells into recipients with sickle cell trait, expressed in per cent of surviving donor's cells.

is too small to establish any definite correlation between the clinical severity of the donor's hemolytic process and the survival time. This problem requires further study, particularly in cases during hemolytic crisis.

DISCUSSION

The average life span of the red cell in dogs and human beings under physiologic conditions is approximately 120 days. This now must be considered as an established fact. By quite different methods, for instance, pigment studies in dogs with renal biliary fistula[9, 10] and survival time determination after transfusion of variously labeled red cells (Ashby technique,[5, 7, 23] sulfhemoglobin,[11] radioactive nitrogen[12]) others have arrived at this same figure. British workers,[7, 13, 14] using the method of differential agglutination, plotted the numbers of surviving cells against time in a diagram and found a linear type of disappearance. Normally a fixed fraction of those cells introduced at transfusion are removed in the unit of time (0.83 per cent per day). This straight line of decay depends on a property (age) of the transfused cells themselves. The finding of such a straight line of disappearance over a period of approximately 120 days may therefore be interpreted as the expression of erythrocyte disintegration by normal means.

If abnormal conditions exist, these two criteria are altered: the survival time is considerably shortened and the graphs are either straight, but steep, or show a curved appearance. Such abnormal lines of decay are indicative of the presence of abnormal hemolytic mechanisms. By means of the cross determination of the survival time one may then gain better insight into the nature of these pathologic conditions. When normal erythrocytes transfused into a recipient with a hemolytic syndrome survive normally, whereas the recipient's own cells transfused into a normal person have a considerably shortened life span, an intracorpuscular abnormality may be suspected. Contrariwise, when normal red cells transfused into a patient with a hemolytic syndrome are as rapidly eliminated as the patient's own cells, an indiscriminating extracorpuscular mechanism can be assumed.

Our experiments demonstrate that trait cells have a normal survival time and an almost straight line of decay when introduced into patients with sickle cell anemia. Anemia cells, however, when transfused into trait carriers, are destroyed much more rapidly, as can be seen from the abnormal graphs. Therefore the pathogenic principle operating in sickle cell anemia resides within the red cells themselves; no extracorpuscular mechanism can be made responsible for this hemolytic syndrome. Altmann[15] and Callender and Nickel[8] reported that the longevity of normal erythrocytes in patients with sickle cell anemia is unaltered. The finding that trait cells also survive normally is of practical importance because these erythrocytes can be used safely for therapeutic transfusions.

Our investigations have established a fundamental difference between the survival time of anemia and trait cells. Since both types of cells show the sickling tendency, the question arises whether this difference is related to a variable degree of the basic abnormality responsible for the sickling process or whether an additional structural alteration of the anemia cell may be involved. The following facts and observations require consideration in the evaluation of this problem.

(1) The well-known clinical fact that one does not encounter transitional types between sicklemia and sickle cell anemia cases would support the existence of an additional pathogenic factor in the production of the anemia.

(2) Hahn and Gillespie,[16] in 1927, made the significant discovery that distortion of susceptible corpuscles into sickle cells will take place only if the hemoglobin contained in the cells is in the reduced state. In vitro studies of the sickle cell phenomenon fail to show any clear distinction between trait and anemia if the sickling test is performed under conditions optimal for the reduction of hemoglobin. Neither with the gas chamber method of Hahn and Gillespie[16] nor with the rapid method of Singer and Robin,[1] who used a broth culture of *B. subtilis* for the reduction of the oxyhemoglobin, could any definite correlation be detected between the number of sickling cells in the preparation or the rapidity and degree of the sickling process and the presence of either trait or anemia. The often demonstrable difference in the results between patients with anemic and trait cells with the moist stasis method[17] is probably caused by the increased number of nucleated red cells, reticulocytes, and white cells present in the anemia blood; only these cells consume oxygen, whereas mature red cells do not. As a proof of this interpretation, the observation[1] may be offered that when trait cells are mixed with the buffy layer of leucemic blood, sickling can almost immediately be elicited, although the unmixed blood may require more than twenty-four hours with the stasis method to achieve this result. On the other hand, intravascular sickling has been observed in anemic patients much more commonly than in trait carriers,[18] and it has been speculated that blood destruction may be due to vascular stasis and obstruction of the capillaries by sickling red cells. Such a pathogenic mechanism, however, is unlikely because it would not account for the demonstrably different survival times of trait and anemia cells in the same patient.

(3) The experiments of Reinhard and co-workers[19] also seem to refute the concept that a more pronounced sickling of cells in vivo may be instrumental in causing the anemia. These workers induced patients with sickle cell anemia to breathe high oxygen concentrations for periods of from eight to twenty days without detecting any inhibition of the rate of hemolysis, although a considerable decrease in the degree of intravascular sickling was noted.

(4) The finding that the pathogenic principle of sickle cell anemia resides within the red cells may lend itself to an explanation of the well-known hemolytic crises which occur in this disorder. Murphy and Shapiro[20] suggested that the age of the red cell exerts an influence on its sickling tendency. According to these authors, normoblasts and reticulocytes are rarely seen in sickled form. This latter statement is not corroborated by our observations when optimal conditions for eliciting the sickling process are maintained. However, one may speculate whether the alterations of the stroma which occur with aging, when superimposed on an already structurally defective cell, do not really lead to the massive disintegration of erythrocytes in a short period of time which is the outstanding hematologic feature of the crisis. After such an episode, the rejuvenation of the blood by many reticulocytes takes place. Then

the same process repeats itself in relation to the average survival time of the cells in the circulation. This concept could be tested by correlating the time intervals between crises with the life spans of the red cells before and after such hemolytic exacerbations.

As can be seen from this discussion, the known facts about the sickling phenomenon do not readily explain the pathogenesis of the anemia. Sickling is the expression of an abnormality of the stroma.[21] If together with this abnormality there exists an additional alteration in the cyto-skeleton,[22] the cell structure becomes much more vulnerable to the vicissitudes of life in the circulation. Thus the survival time of the anemia cell is shortened. We are fully aware that this hypothesis is not yet based on any positive evidence. New experiments must be devised to furnish more factual support for this concept.

SUMMARY

The method of cross determination of the survival time of red cells was used for the study of the pathogenesis of sickle cell anemia. When normal erythrocytes transfused into a recipient with a hemolytic syndrome survive normally, whereas the recipient's own red cells transfused into a normal person have a considerably shortened life span, an intracorpuscular abnormality may be suspected. Contrariwise, when normal red cells transfused into the patient with the hemolytic syndrome are as rapidly destroyed as the patient's own cells, the presence of an indiscriminating extracorpuscular mechanism may be assumed. In this study, trait cells were transfused into patients with sickle cell anemia and anemia cells into healthy recipients harboring the sickle cell trait.

Trait cells survive normally (120 days) when transfused into patients with sickle cell anemia, whereas the patients' own cells continue to be hemolyzed at a faster rate. Anemia cells, when transfused into trait carriers, have a shortened life span with an average of about one-fourth of the normal. Therefore the pathogenic principle operating in sickle cell anemia resides within the red cells themselves; no extracorpuscular mechanism can apparently be made responsible for this hemolytic syndrome.

Trait cells can be used safely for therapeutic transfusions.

An analysis of the known facts about the sickling phenomenon shows that the sickling process which is the expression of an abnormality of the stroma does not lend itself to a satisfactory explanation of the pathogenesis of the anemia. The hypothesis is formulated that sickle cell anemia develops because of an additional alteration in the cyto-skeleton which is qualitatively different from the structural anomaly responsible for the sickling phenomenon.

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INTRAGROUP INCOMPATIBILITY WITH RESPECT TO THE Hr BLOOD FACTORS AS A CAUSE OF MINOR HEMOLYTIC TRANSFUSION REACTIONS

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THE work of Wiener and Peters[1] in demonstrating the role of the Rh factor of Landsteiner and Wiener[2, 3] in the pathogenesis of intragroup hemolytic transfusion reactions now has been amply confirmed.[4-11] This has brought about an important change in the practice of blood transfusion, namely the inclusion of routine Rh testing along with A-B blood grouping tests as a basis for the selection of donors for blood transfusion. The proper application of these tests has led to the virtual elimination of dangerous or major hemolytic reactions as a complication of transfusion. It gradually has become recognized, moreover, that Rh-positive individuals also can be isosensitized, because of the existence of more than one variety of Rh factor and the so-called Hr factors.[12-14] Luckily, these other blood factors are far less antigenic than the original rhesus factor (Rh_0) of Landsteiner and Wiener, so that such cases are quite rare. When isosensitization does occur it hardly ever reaches the degree characteristic of Rh_0 or A-B sensitization, so that the antibodies usually are not demonstrable in the sensitized individual's serum, and the reactions following transfusions of incompatible blood are mild and simulate pyrogenic reactions.[15] These minor hemolytic reactions occurring in Rh-positive recipients are the subject of the present paper.

Paralleling the improvements in transfusion practice brought about by the discovery of the rhesus blood factors, there also has been progress in eliminating pyrogens as a cause of nonspecific chills and fever following blood transfusions. For example, at the Jewish Hospital of Brooklyn, during the period 1945 to 1947, the total number of blood transfusions carried out annually increased from 1,800 to 3,800, yet the number of untoward reactions decreased from fifty-eight to forty-seven, a drop in rate of reactions from 2.6 to 1.2 per cent. This is shown in Table I, together with the figures during a comparable period a decade ago.* The virtual elimination of pyrogenic reactions by meticulous care in preparing the blood transfusion apparatus, together with the prevention of major hemolytic reactions by improvements in blood typing techniques, has served to bring to the fore the other class of reactions, namely the minor hemolytic reactions which form the subject of this report.

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*As will be seen from the Table I, at the present time the bulk of the blood used at the Jewish Hospital of Brooklyn is obtained from the Blood and Plasma Exchange Bank of New York City, so that this improvement is to be credited primarily to the admirable work of Dr. Lester J. Unger who is director of that blood bank which provides many thousand units of blood every month to hospitals throughout the country.

TABLE I. COMPARISON OF INCIDENCE OF TRANSFUSION REACTIONS TODAY AND TEN YEARS AGO

YEAR	TOTAL NUMBER OF TRANSFUSIONS	BLOOD USED (UNITS)	TECHNIQUE OF TRANSFUSION			INCIDENCE OF REACTIONS	
			UNMODIFIED BLOOD (SYRINGE-VALVE)	FRESH CITRATED BLOOD	BANK BLOOD		
			NUMBER OF TRANSFUSIONS			NUMBER	PER CENT
1936-37*	1,209	–	950	259	–	95	7.9
1938*	976	–	360	616	–	22	2.2
1939*	1,213	–	267	946	–	35	2.9
1945	1,884	2,121	2	481	1,638	58	2.6
1946	2,496	2,970	–	581	2,452	50	1.7
1947	3,872	3,978	–	613	3,365	47	1.2

*These data are taken from the paper of Wiener, A. S., Oremland, B. H., Hyman, M. A., and Samwick, A. A.: Transfusion Reactions; Experiences With More Than 3,000 Blood Transfusions, Ann. J. Clin. Path. **11**: 102-121, 1941.

THE Rh-Hr BLOOD TYPES

In early studies on human anti-Rh sera it was soon found that in addition to the type corresponding to the original anti-rhesus serum (now designated anti-Rh_0),[2, 3] which gave 85 per cent positive reactions on Caucasian blood, two other varieties of anti-Rh exist, namely anti-rh′ (70 per cent positive)[3, 4] and anti-rh″ (30 per cent positive).[16, 17] The three anti-Rh sera determine three corresponding factors Rh_0, rh′, and rh″ in human blood, which in combination give rise to eight types of blood instead of two, Rh positive and Rh negative.[18]

TABLE II. SCHEME OF THE EIGHT Rh BLOOD TYPES

BLOOD CONTAINING FACTOR Rh_0 (Rh POSITIVE)				BLOOD NOT CONTAINING FACTOR Rh_0 (Rh NEGATIVE)			
	REACTIONS WITH SERA				REACTIONS WITH SERA		
DESIGNATION OF TYPES*	ANTI-rh′	ANTI-rh″	ANTI-Rh_0	DESIGNATION OF TYPES*	ANTI-rh′	ANTI-rh″	ANTI-Rh_0
Rh_0	–	–	+	rh	–	–	–
Rh_1	+	–	+	rh′	+	–	–
Rh_2	–	+	+	rh″	–	+	–
Rh_1Rh_2	+	+	+	rh′rh″	+	+	–

*Type Rh_1 contains the two factors Rh_0 and rh′, the name Rh_1 being short for Rh'_0; similarly Rh_2 is short for Rh''_0 and Rh_1Rh_2 is short for $Rh'_0Rh''_0$.

Factor Rh_0 is written with a capital letter to indicate that it is the most antigenic and therefore the most important clinically, and also that it has a special genetic position. Factors rh′ and rh″ are written with small letters to conform with their lesser clinical importance and also to indicate that these two Rh factors are on an equal plain genetically and serologically, very similar to the agglutinogens A and B. Thus, considering factors rh′ and rh″ alone, four types of blood are possible which are analogous to the four Landsteiner blood groups. When factor Rh_0 is taken into account, a double scheme of four types each results, as shown in Table II. Thus any one who understands the four blood groups immediately masters the eight Rh blood types.

To account for the heredity of the original Rh_0 factor, Landsteiner and Wiener[3] postulated the existence of a pair of allelic genes, *Rh* and *rh*. With the discovery of the eight Rh types, Wiener[18, 19] found it necessary to postulate

TABLE III. EIGHT Rh TYPES AND THEIR TWENTY-ONE GENOTYPES

Rh BLOOD TYPES	APPROXIMATE FREQUENCY IN N.Y.C. (%)	POSSIBLE GENOTYPES
rh	13.0	rr
rh′	1.5	$r\,r'$ and $r'r$
rh″	0.5	$r''r''$ and $r''r$
rh′rh″	0.01	$r'r''$
Rh_0	2.5	R^0R^0 and R^0r
Rh_1	52.5	R^1R^1, R^1r', R^1r, R^1R^0, and $r'R^0$
Rh_2	15.5	R^2R^2, R^2r'', R^2r, R^2R^0, and $r''R^0$
Rh_1Rh_2	14.5	R^1R^2, R^1r'', and $r'R^2$

the existence of a minimum of six allelic genes, designated as R^0, R^1, R^2, r, r', and r'' respectively. It will be noted that under this system the notations for genes and genotypes are different from the notations for the agglutinogens (or factors) and phenotypes, so that ambiguity is avoided. In Table III are shown the genotypes corresponding to the eight phenotypes, according to Wiener's theory of multiple allelic genes. The accuracy of this theory has been established by studies on a large series of families, as well as by statistical studies on the distribution of the Rh blood types.[10-29*] For example, in Table IV is given a summary of the author's studies[29] carried out on a total of 525 families with 948 children. In these studies only two seeming exceptions to the theory were encountered and both of these were proved to be due to illegitimacy.

TABLE IV. SUMMARY OF AUTHORS' FAMILY-STUDIES TO DATE

MATING	NUMBER OF FAMILIES	NUMBER OF CHILDREN OF TYPES							TOTAL
		rh	Rh_1	Rh_2	Rh_1Rh_2	Rh_0	rh′	rh″	
rh × rh	12	25	0	0	0	0	0	0	25
rh × Rh_1	169	51	221	0	0	16	2	0	290
rh × Rh_2	44	25	0	52	0	1	0	0	78
rh × Rh_1Rh_2	63	(1)	55	51	0	0	0	0	107
rh × Rh_0	7	3	0	0	0	9	0	0	12
rh × rh′	3	1	0	(1)	0	0	2	0	4
rh × rh″	2	2	0	0	0	0	0	5	7
Rh_1 × Rh_1	73	10	124	0	0	5	1	0	140
Rh_1 × Rh_2	37	11	25	11	19	1	3	0	70
Rh_1 × Rh_1Rh_2	48	0	60	12	37	0	0	0	109
Rh_1 × Rh_0	12	5	10	0	0	3	0	0	18
Rh_1 × rh′	8	1	7	0	0	0	2	0	10
Rh_1 × rh″	6	2	3	0	3	0	0	0	8
Rh_2 × Rh_2	3	1	0	5	0	0	0	0	6
Rh_2 × Rh_1Rh_2	14	0	6	14	8	0	0	0	28
Rh_2 × Rh_0	2	0	0	1	0	4	0	0	5
Rh_2 × rh′	4	1	0	2	2	0	2	0	7
Rh_1Rh_2 × Rh_1Rh_2	9	0	4	2	6	0	0	0	12
Rh_1Rh_2 × Rh_0	3	0	2	1	0	0	0	0	3
Rh_1Rh_2 × rh′	2	0	1	1	2	0	0	0	4
Rh_1Rh_2 × rh″	2	0	1	0	1	0	0	0	2
Rh_0 × Rh_0	1	0	0	0	0	1	0	0	1
Rh_0 × rh′	1	0	0	0	0	1	1	0	2
Total	525	139	519	153	78	41	13	5	948

*The alternative between multiple alleles and completely linked genes was discussed a long time ago by the writer and others in relation to the heredity of the A-B-O groups (Wiener, A. S.: Blood Groups and Transfusion, ed. 3, Springfield, Ill., 1943, Charles C. Thomas pp. 185 and 194). The English school have now revived this discussion in relation to the Rh-Hr types.

Levine[30] and Javert[31] demonstrated that Rh-negative blood specimens contain a special agglutinogen designated Hr by them because of its apparently reciprocal relation to Rh. Race and Taylor[32] independently discovered an agglutinogen which they called St, and showed that St was related to rh′ as M is related to N. Wiener and co-workers[33] have proved that Hr and St are identical, so that the designation St has been abandoned. Since the Hr antigen first found was shown by Levine and Fisher to be reciprocally related to rh′, Wiener[34] suggested that it be designated as hr.′ As Fisher[35] pointed out, theoretically three Hr factors are possible, corresponding to the three Rh factors. Actually, hr″ has been found* by Mourant,[36] but convincing evidence regarding the existence of Hr_0 has not yet been published.†

Sensitization to the Hr factors can cause clinical complications similar to those produced by Rh sensitization, namely intragroup hemolytic transfusion reactions and erythroblastosis fetalis. However, the Hr factors are far less antigenic than the Rh factors, so that such cases are quite rare. For the same reason, Hr antisera are much more difficult to procure than Rh antisera, and to date attempts to immunize Hr-negative donors in order to produce such antisera have met with little or no success. Yet Hr antisera are necessary to resolve clinical problems caused by sensitization to factors other than Rh, and for the selection of Hr-negative donors to be used for transfusing patients sensitized to

TABLE V. THE Rh-Hr BLOOD TYPES AND THEIR CORRESPONDING GENOTYPES

Rh BLOOD TYPES†	REACTION WITH SERUM		Rh SUBTYPES†	APPROXIMATE FREQUENCY IN N.Y.C. (%)	POSSIBLE GENOTYPES
	ANTI-hr′	ANTI-hr″			
rh	+	+	rh	13.0	rr
rh′	−	+	rh′rh′	.01	$r'r'$
	+	+	rh′rh	1.0	$r'r$
rh″	+	−	rh″rh″	.005	$r''r''$
	+	+	rh″rh	0.5	$r''r$
rh′rh″	+	+	rh′rh″	.01	$r'r''$
Rh_0	+	+	Rh_0	2.0	R^0R^0 and R^0r
Rh_1	−	+	Rh_1Rh_1	20.0	R^1R^1 and R^1r'
	+	+	Rh_1rh	34.0	R^1r, R^1R^0, and R^0r'
Rh_2	+	−	Rh_2Rh_2	3.0	R^2R^2 and R^2r''
	+	+	Rh_2rh	12.0	R^2r, R^2R^0, and R^0r''
Rh_1Rh_2	+	+	Rh_1Rh_2	14.5	R^1R^2, R^1r'', and $r'R^2$

*Tests which need not be made because the reactions in these combinations are invariably positive, except for types rh′rh″ and Rh_1Rh_2 in the case of individuals carrying the rare genes r^y and R^z. Should these tests be done and negative reactions obtained, there has been an error in technique, and the entire examination should be repeated.

†These are phenotypes and the symbols have been selected so as to indicate what tests have actually been done and what reactions were obtained. For example, type Rh_1 indicates that tests have been made only with sera anti-rh′, anti-rh″, and anti-Rh_0, and positive reactions obtained with anti-rh′ and anti-Rh_0 and a negative reaction with anti-rh″; on the other hand type Rh_1Rh_1 indicates type Rh_1 blood which also has been tested with anti-hr′ serum with which it gives a negative reaction.

*A second example of anti-hr″ has recently been found by Wiener and Peters.[37]

†The theoretical reactions of anti-Hr_0 sera have been presented by Fisher,[35] and also by the writer[34] in previous papers. They are not included in the present paper because the writer has never encountered an anti-Hr_0 serum, though such sera are said to have been found by others (Haberman, S., Hill, J. M., Everist, B. W., and Davenport, J. W., Jr.: Blood, in press).

one of the Hr factors. Another important application of the Hr antisera is for a presumptive test for heterozygosity or homozygosity of Rh-positive husbands of Rh-negative prospective mothers who are known to be sensitized to the Rh factor. Still another application is in disputed paternity cases, since the Rh-Hr tests considerably increase the chances of excluding a falsely accused man.

After a blood sample has been classified into one of the eight Rh blood types with the aid of the three Rh antisera, anti-rh′, anti-rh″, and anti-Rh_0, if indicated the blood can be subtyped with the aid of anti-Hr sera. The Hr tests are done after the Rh tests in order to avoid unnecessary Hr testing, so as to conserve the rare and valuable Hr antisera. Thus, only blood of types Rh_1 and rh′ need be tested with anti-hr′ serum, and similarly, only blood of types Rh_2 and rh″ need be tested with anti-hr″ serum. Other combinations uniformly give positive reactions, as shown in Table V, barring blood from the rare individuals who carry genes r^y and R^z.[38, 39]

The Rh-Hr subtypes are designated as follows:

Rh_1 hr′– is called Rh_1Rh_1.
Rh_1hr′+ is called Rh_1rh.
rh′ hr′– is called rh′rh′.
rh′ hr′+ is called rh′rh.

Similarly,

Rh_2 hr″– is called Rh_2Rh_2.
Rh_2 hr″+ is called Rh_2rh.
rh″ hr″– is called rh″rh″.
rh″ rh″+ is called rh″rh.

It must be emphasized that these designations refer to *phenotypes* not *genotypes*. Thus, corresponding to phenotype Rh_1Rh_1, two genotypes, R^1R^1 and R^1r', are theoretically possible, while corresponding to phenotype Rh_1rh, three genotypes, R^1r, R^1R^0, and $r'R^0$, are possible. Of these five genotypes, R^1R^1 and R^1r are by far the most common, and it will be seen that this is the basis for the selection of the phenotype names, namely to indicate the most likely genotype. It is in this sense that anti-hr′ serum can be used to determine the *probable* genotype of type Rh_1 individuals, and anti-hr″ serum to determine the *probable* genotype of type Rh_2 individuals.

From the foregoing it should be evident, as has been pointed out by Wiener, that the Hr factors hold a serologic and genetic position in the scheme of the Rh-Hr blood types analogous to that of the O factor in the less complicated scheme of the Landsteiner A-B-O groups. Just as the O factor is by far the least antigenic among the A-B-O factors, similarly the two Hr factors are far less antigenic that the three (or four*) Rh factors. In this connection it may be mentioned that clinical sensitization to the O factor has occurred producing fetal erythroblastosis entirely comparable to cases of erythroblastosis caused by Hr sensitization.[42]

*The existence of a fourth Rh factor, rh‴, was predicted by Wiener[40] and confirmed by Callender and Race[41] who designated it C^w.

MATERIALS AND METHODS

The cases forming the basis of this study were derived principally from the Blood Transfusion Division of the Jewish Hospital of Brooklyn, though a number of cases from other institutions were also included. Before every transfusion 10 c.c. of blood were obtained from the patient by venepuncture, 5 c.c. of which were mixed with dried oxalate powder, as for the Wintrobe-Landsberg sedimentation test, and used for the grouping, Rh, and cross-matching tests, while 5 c.c. were placed in a dry clean tube and kept in the refrigerator for future reference. If any patient had a chill or other signs of reaction during or after the transfusion, a second sample of blood was obtained without delay. The group and Rh type of the patient's and donor's blood specimens were verified, and the depth of color of the patient's serum (or plasma) was compared with that of the pretransfusion sample. All glassware, syringes, and needles for these studies were sterilized in the hot air oven in order to avoid hemolysis due to moisture, and care was taken also when separating the clots to avoid artefacts caused by trauma to the clot. If the findings verified the original grouping and Rh report, and if there was no significant change in the color of the patient's plasma, the reaction was usually interpreted as pyrogenic and no contraindication was ordinarily considered to exist to further transfusions.* In cases where the posttransfusion plasma was definitely darker than the pretransfusion sample, further studies were carried out to detect the possible presence of intragroup incompatibilities of an unusual type not provided against by the routine pretransfusion tests.

For simplicity in analyzing the results, the presentation will be confined to a statistical analysis of the groups, M-N types and Rh-Hr types of patients having reactions to transfusions, together with some brief clinical data concerning the reactions, and the results of tests for irregular isoantibodies in the patients' sera.

RESULTS

In Table VI are listed thirty-two cases of posttransfusion febrile reactions. In Cases 1 to 22 there was some clinical evidence of posttransfusion hemolysis, while in Cases 23 to 32 there was no evidence of hemolysis.

It will be seen that the distribution of the A-B-O blood groups and M-N types among the patients did not differ significantly from that occurring in the general population. On the other hand, while the distribution of the Rh-Hr among the control cases (Cases 23 to 32) did not seem unusual, the distribution among the patients who had hemolytic reactions deviated strikingly. One cannot help but be impressed by the large proportion of Hr-negative patients in the series—fourteen Rh_1Rh_1 and three Rh_2Rh_2 out of twenty-two cases. These results suggest that sensitization to the Hr factors, hr′ and hr″, must have played a role in many of the reactions.

Attempts to demonstrate the presence of irregular Hr isoantibodies in the sera of the Hr-negative patients failed in all but three of the cases in this series. In a fourth case (Case 14) which has been reported elsewhere,[14] no Hr antibody was found but instead a different irregular isoantibody reacting with about 14 per cent of all Caucasian blood and not corresponding to any hitherto described antibody.† Of the three cases showing the presence of Hr isoantibodies, in two (Cases 21 and 22) the specificity corresponded to hr′, while in the third (Case

*If any of the blood used for transfusion was left in the transfusion bottle, smears and cultures were made for the possible presence of molds or bacteria. In only rare cases has contamination proved to be the cause of reactions in our series.

†A sample of this serum was sent to Dr. A. E. Mourant who stated that it gave reactions corresponding to his own so-called Kell serum. Since in the original report[12] on Kell antibody the serum was said to give only 7 per cent reactions instead of 14 per cent as obtained by us with anti-Si serum, more work must be done before the two antigens may be assumed to be identical.

TABLE VI. LIST OF CASES WITH FEBRILE REACTIONS TO BLOOD TRANSFUSIONS

CASE	PATIENT'S BLOOD TYPE	OTHER FINDINGS
1	$A_1NRh_1Rh_1$	Rise in icterus index.*
2	$A_1MRh_1Rh_1$	Rise in icterus index.*
3	$A_1Rh_1Rh_2$	Rise in icterus index.*
4	$OMRh_1Rh_1$	Rise in icterus index.*
5	$BMNRh_1Rh_1$	Rise in icterus index.*
6	$A_1MNRh_1Rh_1$	Icterus index rose from 5 to 12 units.
7	$OMRh_2Rh_2$	Rise in icterus index*; ill-defined irregular antibody detectable in serum at times.
8	A_1MNRh_1rh	Icterus index rose from 1 to 8 units.
9	$A_1MNRh_1Rh_1$	Icterus index rose from 10 to 12 units.
10	$A_1MNRh_1Rh_2$	Hemoglobinuria.
11	$ONRh_1Rh_1$	Cyanosis; drop in hemoglobin.
12	$ONRh_1Rh_1$	Icterus index rose from 2 to 4 units.
13	$OMNRh_1rh$	Icterus index rose from 2 to 4 units.
14	$AMNRh_1Rh_1$	Shock; drop in hemoglobin concentration; irregular isoantibody, anti-Si, demonstrable by conglutination technique.
15	$A_2NRh_1Rh_1$	Rise in icterus index from 2 to 3 units.
16	$OMRh_2Rh_2$	Rise in icterus index.*
17	$BMNRh_2$	Rise in icterus index.*
18	$OMRh_2Rh_2$	Strong anti-hr″ agglutinin demonstrable in patient's serum.
19	$A_1MRh_1Rh_1$	Collapse, severe hemoglobin urea.
20	$OMNRh_1Rh_1$	Rise in icterus index from 4 to 12 units; dark urine.
21	$A_1BMNRh_1Rh_1$	Strong anti-hr′ agglutinin demonstrable in patient's serum.
22	$BMNRh_1Rh_1$	Patient's blood showed evanescent rise in hemoglobin concentration, then a progressive fall terminating with patient's death; anti-hr′ agglutinins in patient's serum.
23	$ONRh_1Rh_2$	No change in icterus index.†
24	$AMNRh_1rh$	No change in icterus index.†
25	ORh_1rh	No change in icterus index.†
26	$OMNRh_1rh$	No change in icterus index.†
27	$ONRh_1Rh_2$	No change in icterus index.†
28	$A_1MNRh_1Rh_2$	No change in icterus index.†
29	A_1NRh_1rh	No change in icterus index.†
30	A_1NRh_1rh	No change in icterus index.†
31	$OMRh_1Rh_2$	No other data.
32	$OMRh_1rh$	No other data.

*The posttransfusion plasma was darker to the eye than the pretransfusion plasma, but no actual measurement of the icterus index was made.

†The depth of color of the pre-and posttransfusion plasmas appeared the same to the eye.

18) the specificity proved to be hr″. This case of hr″ sensitization was the first with demonstrable antibodies of this specificity to be encountered since the original report of Mourant,[36] and therefore has been reported in detail elsewhere.[37] With regard to the two cases of hr′ sensitization with demonstrable antibodies, the scanty clinical data available concerning these patients are presented below for the first time.

CASE 21.—This patient was brought to our attention by Dr. William Thalhimer who had encountered difficulty in finding compatible blood for transfusing the patient because of the presence of an irregular isoagglutinin. The patient was being treated for a spinal cord tumor.*

The patient had had two normal children; the 20-year-old son and 16-year-old daughter were both well. She never had had any miscarriages or stillbirths.

On Feb. 17, 1939, the first exploratory operation had been performed. According to the patient, she received her first blood transfusion in 1945. During that year the patient received three transfusions, which were followed by chills. (The patient was not sure whether all the transfusions were followed by reactions.)

During the hospital admission of this study, Nov. 18, 1947, the patient was given a transfusion of 500 c.c. of group AB, Rh-positive blood, following which she had a mild chill.

*At the Montefiore Hospital, Bronx, N. Y.; we are indebted to Dr. H. M. Zimmerman for his cooperation in obtaining additional blood for study.

A second transfusion, this time of 300 c.c. of group A, Rh-negative blood, was given Nov. 20, 1947, and this was followed by a severe chill. A blood count done on Nov. 14, 1947, showed a hemoglobin concentration of 9.5 Gm. per 100 c.c.; R.B.C., 3.30 million per cubic millimeter; W.B.C., 14,100 per cubic millimeter; polymorphonuclears, 76 (six band forms); lymphocytes, 23; and monocytes, 1. A repeat blood count of Dec. 10, 1947, showed a hemoglobin concentration of 10 Gm. per 100 c.c.; R.B.C., 3.70 million; W.B.C., 7,600; polymorphonuclears, 70 (four band forms); lymphocytes, 25; monocytes, 5.

Our own studies on the patient's blood showed her to belong to group AB, subgroup A_1B, type MN, and type Rh_1Rh_1. Since the patient was Hr negative (or more exactly, hr′ negative), this suggested that the irregular antibody in the serum might be an anti-hr′ agglutinin. In fact, titrations of the patient's serum at body temperature proved that a powerful anti-hr′ agglutinin was present, the titer of the serum for homozygous hr′-positive blood (for example type rh) being approximately 250 units, while the titer for heterozygous hr′-positive blood (for example type Rh_1rh) was about half as high. However the serum exhibited a tendency to clump most if not all blood specimens of type Rh_1Rh_1, particularly when the mixtures were allowed to cool. Titrations at refrigerator temperature showed that this was due to the presence of a strong autoagglutinin. This interfering antibody could be removed by repeated absorptions with type Rh_1Rh_1 of any blood group, and in that way it was possible to obtain a satisfactory anti-hr′ reagent for subtyping Rh_1 and type rh′ individuals. Some of the tests indicated the possible presence of still a third abnormal antibody in the patient's serum, and, while this antibody was not studied carefully, in view of the patient's subgroup (A_1B) the likelihood is that its specificity was anti-O.

CASE 22.—An intern at a hospital in New York City consulted us concerning the following transfusion problem.

The patient, a woman, was said to have pernicious anemia with cardiac complications. The pregnancy history was not known, but the patient was said to have been transfused several times approximately a year previously. On admission to the hospital on Nov. 7, 1947, the patient's condition was critical, the blood having a hemoglobin concentration of only 2 Gm. per cent, with R.B.C. 1.1 million per cubic millimeter and 0.4 per cent reticulocytes on the smear. The patient was given an immediate transfusion of a total of 1,500 c.c. of blood and placed on liver therapy. A blood count of November 10 showed that the hemoglobin concentration had risen to 8 Gm. per cent, with R.B.C. 2.4 million and 0.4 per cent reticulocytes on the smear. By November 12 the hemoglobin concentration had fallen to 7 Gm. per cent with R.B.C. 1.48 million, though there were now 4.8 per cent reticulocytes on the smear; at this time the icterus index was found to be 32 units. By the next day the hemoglobin concentration had dropped to only 4 Gm. per cent with a red blood cell count of 1.0 million, while the icterus index rose to 75 units. The patient was then given another transfusion of 500 c.c. of blood, this time without any reaction. The following day the patient was clinically jaundiced and blood was referred to us for study.

In the blood typing tests the following reactions were obtained.

REACTIONS WITH ANTISERA OF SPECIFICITY

A	B	M	N	rh′	rh″	Rh_0	hr′
–	++±	++±	$\frac{1}{10}$(++)	++	$\frac{1}{10}$(+)	++±	$\frac{1}{10}$(++)

These results indicated that the patient belonged to group B, type M, type Rh_1Rh_1 but that her body also contained some group B, type MN, type Rh_1Rh_2 blood, probably remaining from the transfusion received the day before. This led us to entertain the likelihood that a developing sensitization to the hr′ factor was the cause of the rapid elimination from the patient's body of the 1,500 c.c. of blood she had received the week previously. Examination of the patient's serum did reveal the presence of an irregular agglutinin,

most active at body temperature, which did not clump her own blood cells or blood from other type Rh_1Rh_1 individuals of groups B and O, confirming the prediction that she was sensitized to the hr′ factor. The hr′ agglutinin was weak, however, so that while blood specimens homozygous for the hr′ factor, for example, types rh and Rh_2, were clumped up to dilution 1:4, blood heterozygous for the hr′ factor, for example, types Rh_1rh and Rh_1Rh_2, was not clumped or only feebly clumped. The low titer of the hr′ antibody explained the continued presence in the patient's circulation of some of the type Rh_1Rh_2 blood received by transfusion the day before.

In view of these findings it was advised that for further transfusions to this patient only hr′-negative (type Rh_1Rh_1) group B blood be used. Unfortunately the patient died before another transfusion could be given.

The cases listed in Table VI, and especially the two cases just described, demonstrate that Hr sensitization may pose a serious problem where patients require repeated transfusions over a long period of time. As has been demonstrated elsewhere,[46, 47] the wide spacing of the transfusions is probably the most important factor in the development of isosensitization. For example, two or more transfusions spaced widely apart, as in the cases just described, are much more likely to induce sensitization than a large series of transfusions given within a short period of time. In fact this principle has been applied successfully in the production of anti-Rh sera of specificities Rh_0, rh′, and rh″, though we have not yet succeeded in producing anti-Hr sera in this manner. Two of our type rh donors,[48] both of group AB, type N, formed agglutinins of specificity anti-M as well as antibodies of specificities Rh_0 and rh″ when injected with $OMRH_2Rh_2$ blood.[50] These observations lead one to draw certain analogies between the three systems of blood group and type antigens as shown in Table VII.

TABLE VII. ANALOGIES AMONG A-B-O, M-N AND Rh Hr SYSTEM OF ANTIGENS*

SYSTEM	MAJOR AGGLUTINOGENS (STRONGLY ANTIGENIC)	MINOR AGGLUTINOGENS (WEAKLY ANTIGENIC)
A-B-O	A, B, C,† A_1	O (A_2)‡
M-N	M	N
Rh-Hr	Rh_0, rh′, rh″	hr′, hr″§

*For simplicity the rarer types A_3, M_2, N_2 and so on are not included in this table.
†The common antigen shared by blood containing agglutinogen A or B or both.
‡The antigen shared by agglutinogens O and A_2 but absent from agglutinogens A_1 and B.
§Convincing evidence concerning the existence of the theoretically possible antigen Hr$_0$ has not yet been published.

Recently we were consulted concerning a case which offered an unusual problem of interest in connection with the subject under discussion. The patient was a boy requiring repeated transfusions over a period of years, and we were asked for advice in the selection of donors in order to avoid, if possible, the development of isosensitization. Grouping and Rh-Hr tests on this patient and the parents gave the results shown in Table VIII. As can be seen, this patient offered a problem practically impossible to solve. Since he belonged to type rh′, his blood lacked the Rh_0 agglutinogen and so he had to be given Rh-negative blood. At the same time, he was hr′ negative, and it was desirable also to avoid the possibility of Hr sensitization. Unfortunately the only type of blood which is both Rh_0 negative and hr′ negative is blood of the rare type rh′rh′ identical

TABLE VIII

BLOOD OF:	GROUP	M-N TYPE	Rh-Hr TYPE	
			PHENOTYPE	GENOTYPE
Father of patient	A_1	N	Rh_1Rh_2	$r'R^2$
Mother of patient	A_1	MN	Rh_1Rh_1	R^1r'
Patient	A_1	N	rh'rh'	$r'r'$

with the patient's blood and with an incidence of only 1 in about 10,000 individuals. In addition, the patient belonged to type N, which has a frequency of 20 per cent, so that a completely compatible donor, even using group O donors, would occur only once among about 50,000 individuals. In this case, therefore, we had to be content to take into account only the most antigenic of the three factors Rh_0, hr′, and M, and so ordinary type rh donors of group A were used. Incidentally, the case offers an interesting example of the genetics of the Rh-Hr types as shown in Table VIII.

COMMENT

Our attention was first called to the possibility of hemolytic transfusion reactions caused by isosensitization to the Hr factor by a patient seen in 1944. In this case, reported by Speck and Sonn,[44] the patient, who belonged to type Rh_1Rh_1 and group AB, had a hemolytic reaction during a post-partum transfusion of type rh, group AB blood. However, as in most of the cases described in this paper, abnormal isoantibodies were not clearly demonstrable in the patient's serum. More recently Sussman[45] has reported a case of hr′ sensitization by transfusion in which the patient's serum contained strong isoantibodies which, however, were demonstrable only by the conglutination technique.

In view of our findings it is necessary for all hospitals giving large numbers of transfusions, especially to patients requiring repeated transfusions spread over a long period of time, to have available not only type rh but also type Rh_1Rh_1 and type Rh_2Rh_2 donors, at least of group O, for use for isosensitized patients. For the present it is still not practicable to carry out Hr tests on all patients requiring transfusions for selecting donors. Since the Hr factors are only poorly antigenic, we ordinarily disregard them in the pretransfusion tests. Only when a patient has had a reaction do we test for the Hr factors, and if such a patient is found to belong to type Rh_1Rh_1 or type Rh_2Rh_2, the precaution is taken to use only Hr-negative donors for future transfusions. Ideally, however, the tests should eventually become part of the routine pretransfusion selection of donors, not only to prevent reactions but also to prevent sensitization, especially of women who might subsequently have children with erythroblastosis caused by Hr sensitization.[49]

SUMMARY

The introduction of routine Rh testing along with blood grouping tests as a basis of the selection of donors for transfusions has served to eliminate the danger of serious hemolytic reactions. Simultaneously there has been a reduction in the frequency of posttransfusion chills, as a result of the perfection of methods of eliminating pyrogenic materials from blood transfusion apparatus. Thus at the author's institution the frequency of febrile reactions dropped from

7.9 per cent in 1936 to 2.9 per cent in 1939 and to only 1.2 per cent by 1947. The virtual elimination of pyrogenic reactions has served to make more prominent another class of hemolytic reactions, usually only of minor severity, occurring in Rh-positive patients becoming sensitized by repeated transfusions given over a long period of time.

In a series of twenty-three Rh-positive patients having febrile reactions and at the same time showing evidence of posttransfusion hemolysis, as many as seventeen were Hr negative (fourteen Rh_1Rh_1 and three type Rh_2Rh_2), while among ten patients with febrile reactions but without evidence of hemolysis none were Hr negative. This indicates that Hr sensitization plays a predominant role as a cause of hemolytic reactions in Rh-positive patients. Hr antibodies were clearly demonstrable in the sera of only three (two anti-hr′ and one anti-hr″) of the seventeen presumably sensitized patients. This indicates that Hr sensitization, when it occurs at all, is usually mild in degree. This conforms with the usual mild course of reactions caused by Hr sensitization in that such reactions are usually so harmless that they are passed off as ordinary pyrogenic reactions. However, that such reactions may sometimes endanger the life of the patient is demonstrated by the fact that one of the three patients with demonstrable antibodies died.

While routine pretransfusion Hr typing is still not practicable, one should at least investigate every febrile reaction for evidence of hemolysis. If hemolysis has occurred even though the patient is Rh positive, Hr tests should be done, and if the patient is found to be Hr negative, only Hr-negative blood of a compatible blood group should be used for future transfusions. If Rh-negative patients have reactions despite transfusions of type rh blood, one should search for other sensitizations, particularly against the M factor. Particularly difficult to solve will be instances of multiple sensitization, one of the most common examples of which, in the author's experience, is double sensitization to factors Rh and M.

Eventually blood transfusion practice should include suitable precautions to avoid sensitization against the Hr factors as well as the Rh factors, particularly in the case of women, in order to avoid the birth of babies with erythroblastosis caused by Hr sensitization.

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INFECTIOUS HEPATITIS INADVERTENTLY TRANSMITTED WITH THERAPEUTIC MALARIA

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ACUTE hepatitis resulting from the transmission of an icterogenic agent in blood and blood products has been described frequently in recent years. The purpose of this paper is to call attention to a particular mode of transmission. It has been recognized that an icterogenic agent might possibly be transferred when patients are inoculated with therapeutic malaria. However the hazard has not received sufficient emphasis to cause suitable precautions to be taken in many hospitals using this type of fever therapy. The menace is particularly great since the usual efforts to perpetuate a strain of malaria also mean serial transmission of the icterogenic agent.

The icterogenic agent has been shown to be a virus.[2] Havens[10] has presented evidence that the icterogenic agents of infectious hepatitis and homologous serum jaundice are different viruses. He bases his argument primarily on the fact that the incubation period of the former is 15 to 34 days and of the latter, 58 to 134 days. In 1943 Beeson[1] reported seven cases of jaundice occurring one to four months following transfusions. Other authors[5] have reported a mean incubation period in homologous serum jaundice of 100.1 days. According to the criteria of Havens, the cases to be presented in this paper would be classified as infectious hepatitis rather than homologous serum jaundice. The most common sources of infection have been contaminated lots of yellow fever vaccine,[2, 4, 5] pooled plasma,[6-8] and mumps convalescent plasma.[3] In 1947 Chalmers[9] reported on 450 patients who had been given therapeutic malaria. Of this group, thirty-six cases developed jaundice and evidence of liver damage. He felt that his cases were suggestive of the transfer of an icterogenic agent at the time of inoculation with malaria. In Chalmers' series ninety-nine patients were mosquito-inoculated and none of them developed jaundice.

We shall describe in this paper six patients who received serial inoculations of the same strain of quartan malaria and subsequently developed severe jaundice and marked evidence of hepatocellular damage. These cases were discovered when one of the convalescing patients was received by one of the authors in an intrahospital transfer; consequently, full study of these patients was undertaken only after they had begun the clinical recovery phase. For this reason all the laboratory data that might be desired were not obtained during the acute phase of the illness. The patients had been given malarial fever therapy in treatment of central nervous system syphilis. They were all men whose ages ranged from 29 to 51 years, and all were considered good risks for fever therapy with malaria.

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Prior to inoculation there was no clinical evidence of liver disease, and no hepatotoxic drugs were given during hospitalization. The incubation period in this series ranged from 20 to 43 days, with an average of 32.5 days. The estimated incubation period in some instances is probably longer than the actual period since the patients rarely expressed any subjective symptoms. The sequence of, and the time interval between, the inoculations will be stressed in the case reports. The results of the bromsulfalein tests were obtained by using the 5 mg. per kilogram dose. The standard technique for inoculation with malaria at this hospital has been to give 5 c.c. of donor blood intravenously and 5 c.c. intramuscularly.

REPORT OF CASES

CASE 1.—This patient (P. W.) was probably the original source of the virus in this series. All persons who had received this strain of malaria previously showed no evidence of infectious hepatitis. The six subsequent patients inoculated did develop marked evidence of infectious hepatitis. In later questioning P. W. admitted that two years earlier in the South Pacific several of his tent mates had developed jaundice and that he had had an episode of malaise with vague abdominal distress. He had had no further symptoms until the present illness. The patient was a 34-year-old white man who was inoculated with malaria May 5, 1947. His fever was terminated June 29, 1947, after fifty-two hours above 104°. At no time was jaundice noted nor were symptoms of hepatitis present. During the course of fever the red blood cell count fell from 5,210,000 to 4,100,000 per cubic millimeter. The cephalin flocculation test 139 days after inoculation was positive plus 4 after twenty-four hours, and on the same date the bromsulfalein test showed 9.5 per cent retention after thirty minutes. The patient has continued free of any hepatic symptoms.

CASE 2.—J. I., a 41-year-old white man, was inoculated May 16, 1947, with blood from P. W. (Case 1). The patient complained of malaise and anorexia forty-one days after inoculation, and on July 5, 1947, jaundice and symptoms of hepatitis had become so severe that the malaria was terminated. During the course of fever, the red blood cell count fell from 5,400,000 to 3,230,000. A leucopenia did not develop. On the forty-eighth day following inoculation the icterus index was 100 units and serum protein was 8.25 Gm. per 100 c.c., with albumin 3.45 Gm. per 100 c.c. and globulin 4.8 Gm. per 100 cubic centimeters. The temperature curve did not fall to the base line between the spikes of fever, and after the malaria was terminated a low grade temperature elevation continued for twenty days.

CASE 3.—F. S., a 40-year-old white man, was inoculated with malaria on July 2, 1947, with blood from J. I. (Case 2). He complained of nausea and vomiting seventeen days after inoculation, and two days later marked jaundice appeared. Abdominal pain was present and there was tenderness at the right costal margin though the liver could not be palpated. His fever was terminated twenty-six days after inoculation. The red blood cell count did not fall during the fever therapy, possibly because of dehydration. Between spikes of fever due to malaria the temperature curve did not return to the base line. The icterus index twenty-one days after inoculation was 90 units, at seventy-seven days, 15.9 units, and at 109 days, 13 units. The cephalin flocculation test seventy-seven days after inoculation was positive plus 4 at the end of twenty-four hours and thirty days later had become negative. The bromsulfalein test eighty-five days after inoculation showed 37 per cent retention at the end of thirty minutes.

CASE 4.—W. B., a 51-year-old white man, was inoculated with malaria on July 29, 1947, with blood from F. S. (Case 3). Jaundice appeared thirty-eight days after inoculation and termination of fever was begun. The patient had twenty-six hours of fever above 104°. The initial red blood cell count of 4,880,000 and hemoglobin of 13.2 Gm. fell during fever to 3,900,000 and 9.2 Gm. respectively. The white blood cell count was never above 7,000 nor

below 5,400. The bromsulfalein test ninety-four days after inoculation showed thirty per cent retention at the end of thirty minutes. Seventy-five days after inoculation the icterus index was 9.6 units and the cephalin flocculation was positive plus 4 after twenty-four hours. At 111 days after inoculation cephalin flocculation was still positive plus four after twenty-four hours.

CASE 5.—C. H., a 39-year-old white man, was inoculated with malaria on July 29, 1947, with blood taken from F. S. (Case 3). Jaundice appeared forty-three days after inoculation and was accompanied by severe abdominal pain, eructation, nausea, and vomiting. These symptoms were so severe that termination of malaria was necessary forty-eight days after inoculation. During this illness the red blood cell count fell from 4,550,000 to 3,530,000, and the hemoglobin from 13.2 to 10.6 grams. A leucopenia did not develop in this patient. Bed rest was required for three weeks after malaria was terminated. The bromsulfalein test showed 25 per cent retention at thirty minutes seventy-three days after inoculation. Seventy-five days after inoculation the icterus index was still 16.7 units and cephalin flocculation was positive plus 4 after twenty-four hours.

CASE 6.—R. T., a 34-year-old white man, was inoculated with malaria from F. S. (Case 3) on July 29, 1947. Jaundice appeared twenty-seven days after inoculation, and the patient appeared acutely ill with sustained fever and gastrointestinal distress. The fever did not fall below 101° between the malaria spikes. Malaria was terminated thirty days after inoculation. At the peak of the acute illness, the previously normal red blood cell count and hemoglobin fell to 3,590,000 and 13.2 Gm. respectively. The white blood cell count fell to 3,800. Sixty-three days after inoculation the cephalin flocculation was positive plus 4 at the end of twenty-four hours. Sixty-six days after inoculation the bromsulfalein test showed 29.5 per cent retention at the end of thirty minutes, and cephalin flocculation was positive plus 2 at the end of forty-eight hours. Cephalin flocculation was negative after forty-eight hours 117 days after inoculation.

CASE 7.—W. S., a 43-year-old white man, was inoculated with malaria from C. H. (Case 5) on Sept. 5, 1947. Jaundice appeared twenty-seven days after inoculation and the patient complained of abdominal pain, anorexia, nausea, and vomiting. The fever curve rarely fell below 100° between the malaria spikes. A tender liver edge was palpated 3 cm. below the right costal margin. Termination of malaria was necessary thirty-one days after inoculation. The patient had a long convalescence with continuation of gastrointestinal symptoms. Before inoculation with malaria the red blood cell count was 5,210,000 and the hemoglobin was 16.4 grams. During the acute phase of illness the red blood cell count fell to 3,280,000 and the hemoglobin to 10.6 grams. The white blood cell count remained normal. A low-grade fever continued for three weeks after termination of malaria. Thirty-five days after inoculation there was 39 per cent bromsulfalein retention after thirty minutes. Thirty-eight days after inoculation the icterus index was still 14.3 units. The cephalin flocculation test was positive plus 2 at forty-eight hours, seventy-one days after inoculation.

COMMENT

The consecutive development in these patients of evidence of hepatitis which followed serial inoculation with malaria is significant. The fact that all the findings could have been caused by a virulent strain of malaria is acknowledged. However, this same strain of malaria had been used on a great number of other individuals in this hospital without such ill effects. It is felt that the only logical explanation is that an icterogenic agent was transferred at the time of inoculation with malaria. According to Havens' classification, the short incubation period in this series is more compatible with infectious hepatitis than with homologous serum jaundice. The transfer of an icterogenic agent, therefore, constitutes an additional hazard in malarial fever therapy. In addition to the

obvious danger to the patient, this complication necessitates early termination of the fever. The patient who can be a source of the icterogenic agent should not have his blood passed to the next patient, and will probably be a bad risk for fever therapy with malaria as well. It would seem that the best way to avoid this hazard is to perform liver function tests routinely before fever therapy is begun.

SUMMARY

Six patients are presented who consecutively developed evidence of hepatitis following inoculation with therapeutic malaria. An additional patient is described who probably served as the original source of the icterogenic agent in this series. It is believed that infectious hepatitis was inadvertently transmitted with therapeutic malaria.

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STUDIES IN HODGKIN'S SYNDROME

VII. Nitrogen Mustard Therapy

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THE occurrence of leucopenia in individuals exposed to nitrogen mustard gas was recognized during World War I.[1] It was not until World War II however that extensive studies concerning the cytotoxic properties of nitrogen mustard compounds were undertaken. A specific affinity of the *bis* and *tris* (β-chloroethyl) amines for rapidly growing and regenerating tissue in general and a particular susceptibility of the hematopoietic and lymphoid systems to the cytotoxic action of these substances have been described.[1, 2, 11] The therapeutic trial of methyl-*bis* and *tris* nitrogen mustard compounds in lymphomatous and certain other neoplastic diseases followed. Gilman and Philips[3, 10] and Rhoads[4] have summarized the historical background as well as the chemical, pharmacologic, and physiologic properties of this group of chemical agents.

Clinical studies using the *tris* and the *bis* (β-chloroethyl) amines have been carried on by a number of investigators. Patients with a variety of neoplasms exclusive of the hematologic dyscrasias have been treated with these compounds but results in most cases do not appear to justify their continued use. The most encouraging results described to date have been observed in Hodgkin's disease.[12-15] It is generally agreed, on the basis of preliminary published data, that remissions which are induced by this therapy are temporary, lasting 0 to 8 months, and that the clinical results achieved are comparable in many ways to those produced by roentgen rays.[5-9, 12, 14, 15] Karnofsky has restated an opinion shared by many investigators[9] that roentgen therapy is advisable in early localized Hodgkin's lesions and local extensions, and that nitrogen mustard may be more effective in generalized Hodgkin's disease, early and late, and in cases characterized by severe systemic intoxication.

The only persistent systemic toxic effect of the halogenated amines reported at so-called therapeutic dose levels is a more or less severe but transitory damage to hematopoietic and lymphoid tissues. Leucopenia, thrombocytopenia, and a moderate anemia appear in a large percentage of cases under these conditions. A decrease in the number of formed elements in the peripheral blood becomes apparent three to four days after the beginning of therapy and continues for approximately three weeks. Serial bone marrow studies have revealed progressive destructive hypoplasia consistent with the peripheral blood findings. Dur-

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ing the fourth week regeneration takes place.[6, 8, 12] The first cellular elements to be depressed are the lymphocytes, followed in a few days by an absolute panleucopenia with a decrease in the number of polymorphonuclear leucocytes.[5]

Jacobson and associates[6] have described a left shift in the lymphocytes with the appearance of abnormal forms. Similar qualitative abnormalities have been noted in the monocyte series. Both cell types return to normal within two or three weeks. The reticulocytes of the peripheral blood are reduced during the first week to less than 0.1 per cent, and thrombocytopenia develops during the third week after treatment. However, in spite of this obvious and extensive damage to the bone marrow, the great majority of patients revert promptly to a relatively normal hematologic equilibrium within a few weeks after therapy, depending on the degree of normality of the marrow before therapy was instituted. Folic acid, Pentnucleotide, iron, and whole blood do not ameliorate the toxic effects produced.

The present report is based on the treatment of thirty-one patients with Hodgkin's disease with forty-four courses of the methyl-*bis* (β-chloroethyl) amine hydrochloride. In every case the biopsy diagnosis of Hodgkin's disease was confirmed by two or more pathologists before treatment was begun. Two patients received three, nine received two, and twenty received one course of therapy. For the most part the choice of patients in this series followed the criteria of Karnofsky[9]; in addition, patients were selected who presented evidence of local extensions of the disease and who had not responded to roentgen therapy. Serial peripheral blood studies were made in all patients, supplemented whenever practicable by sternal marrow examinations. The supravital staining technique was used in all differential blood cell estimations.

Each patient was given 0.1 mg. of HN_2 per kilogram body weight for five days with the exception of the seven individuals who were given a double dose on two consecutive days and a single dose on the third day. Each dose was dissolved in 10 c.c. of normal saline and injected immediately via the tube through which normal saline was being administered intravenously to the patient. From 200 to 500 c.c. of saline were infused during each injection of HN_2.

In evaluating the results obtained after nitrogen mustard therapy, a remission was defined as a decrease in unfavorable symptoms ascribed to the disease and an objective regression of the disease as measured by roentgenologic and hematologic findings and physical examination.

RESULTS

A summary of the results is presented in Table I. The average remission time following HN_2 in this series was 2.8 months. The longest remissions were four, five, and six months respectively. Some of these cases are still in remission at the time of writing. Ten patients included in this series have expired. The remainder have been maintained on subsequent courses of nitrogen mustard and/or radiation therapy.

An analysis of the results of treatment was made with special reference to the therapy used previous to the initial course of mustard, the specific indica-

TABLE I

CASE AND PATIENT	COURSE OF THERAPY	DURATION OF ILLNESS	PREVIOUS X-RAY THERAPY	NUMBER OF DOSES	LENGTH OF TIME OF REMISSION (MO.)	NAUSEA AND VOMITING	SKIN ERUPTION	ANEMIA	PER CENT CHANGE RETICULOCYTE	LEUCOPENIA	THROMBOCYTOPENIA	BONE MARROW: HODGKIN'S INVOLVEMENT	BONE MARROW: HYPOPLASIA AFTER THERAPY	APPEARANCE OF BLOOD EFFECT (DAYS)	SUPPLEMENTAL THERAPY: WHOLE BLOOD	SUPPLEMENTAL THERAPY: FOLIC ACID	EXPIRED
1 P.D.	1	3 yr.	+	5		+	+	0	2.4-0.2	++	0			33	0	0	
	2			5		+	+	+	2.4-1.0	++++	++			18	+	+	
	3			3		+	+	++++	5.2-2.6	+	++++			25	+	0	+
2 V.V.	4	2 yr.	+	5	2	+	+	+	2.2-0.6	0	++			7	0	+	
3 A.H.	5	5 mo.	+	5	1	+	+	+	1.8-2.0	0	+++	+	+	23	0	+	+
4 N.L.	6	2 yr.	+	5		+		++++	0.2-6.8	0	+			40	0	0	
	7			5		+		++		0	+++				+	0	+
5 O.S.	8	2 yr.	+	5		0		0		0	+				0	0	
	9			5	2	0	+	+		0	+				0	0	
	10			5	1	+	+	0		0	+				0	0	
6 E.F.	11	2 yr.	+	5		+		+		0	++			29	0	0	
7 C.M.	12	6 yr.	+	5	5	+		0	0.8-2.4	+	+			18	0	0	
8 F.N.	13	4 yr.	+	5	6	+		0		++++	++++			22	0	0	
9 M.G.	14	1 yr.	+	5	2	+	+	0	2.2-0.0	0	+			25	0	0	
	15			5		+	+	+++		++++				11	+	+	
10 C.L.	16	3½ yr.	+	5	4	0		0		++	++++			18	0	0	
11 D.R.	17	3 yr.	+	5	1	0	+	0	0.2-0.2	++++	++++		+	10	+	+	+
12 C.E.	18	2½ yr.	+	5	2	+	+	++++	2.2-1.6	+++	+++			35	0	0	
	19			5		+	+	+	0.2-0.0	++++	++++			15	+	+	+
13 L.B.	20	2 yr.	+	5		+									0	+	

14 G.T.	21	2 yr.	+	5	3	0	+	+	1.4-0.4	++++	+++		+	16	+	0	
	22			3	1	0	+								0	+	
15 L.F.	23	6 mo.	+	4		+	+	0	2.4-0.8	0	0			8	0	0	+
16 H.B.	24	7 yr.	+	5	1	+	+	++++	3.0-1.8	++++	+++			30	+	+	
	25			5	2	+	+	++++	1.8-0.8	++++	+++			30	+	0	
17 R.M.	26	4 mo.	+	5		+		+	2.4-1.6	++++	+++	+	+	22	+	0	+
18 M.M.	27	6 yr.	+	5	2	+	+	0	0.2-0.0	+++	0			9	+	0	
	28			5		+	+	++++	4.2-1.2	++++	+++			15	+	0	
19 E.R.	29	2 yr.	+	5		+	+	0	0.4-0.0	+++	++			5	+	0	+
	30			5		+	+	+	2.0-0.2	++++	++			8	+	0	
20 E.C.	31	3 mo.	+	5	2	+		+	1.6-0.2	0	0			12	0	0	
21 H.G.	32	1½ yr.	+	5		+		++	10.8-2.8	++++	+++			12	+	0	+
22 W.M.	33	3 yr.	+	5	3	+		0	1.4-1.6		+		+	5	+	+	
23 R.H.	34	4 yr.	+	5	2	+									0	+	
	35			3	2	+		+	0.8-0.2	+			+	35	0	0	
24 W.R.	36	2 yr.	+	5	3	+		++	1.4-1.4	++				30	0	+	
25 E.H.	37	7 mo.	+	5	2	+		0	6.2-3.2	0	+		+	21	0	0	
26 R.S.	38	3 mo.	+	5		+									0	+	
27 S.M.	39		+	5		+									0	0	
28 M.T.	40	4 yr.	+	5		+	+	++	6.4-1.4	++++	++++			15	+	+	
	41			4		+	+	++++	5.0-0.8	++++	++++			7	+	0	+
29 E.C.	42	8 mo.	0	5	1	+		++		0	++		+	20	0	0	
30 E.S.	43	1 yr.	+	5	1	+							+		+	+	
31 F.P.	44	9 yr.	+	3	1	+		++++	1.6-0.0	++++	+++	+	+	16	+	0	

Anemia: 0, above 4.0 R.B.C.; +, 3.0 to 4.0; ++, 2.5 to 3.0; +++, 2.0 to 2.5; ++++, below 2.0. Leucopenia: 0, above 2,500 W.B.C.; +, 2,000 to 2,500; ++, 1,500 to 2,000; +++, 1,000 to 1,500; ++++, below 1,000. Thrombocytopenia; 0, above 500,000 platelets; +, 300,000 to 500,000; ++, 100,000 to 300,000; +++, 50,000 to 100,000; ++++, below 50,000.

Summary of Table I: Number of cases, 31; number of courses, 44; previous x-ray therapy, 30 cases; average length of remission, 2.8 months; nausea followed by vomiting, 38 courses; skin eruptions followed, 23 courses; anemia below 3.0 million R.B.C., 14 courses; reticulocytosis before therapy, 24 courses; decrease in reticulocytes after therapy, 24 courses; leucopenia below 2,000 W.B.C., 22 courses; thrombocytopenia below 100,000 platelets, 17 courses; average time of appearance of blood depression, 20 days.

tions for HN_2, and the subsequent activity and treatment of the disease. The case numbers used refer to Table I. The patients who obtained benefit from nitrogen mustard therapy were divided into four groups. The first group includes one patient who had received no previous therapy of any kind (Case 29) and those patients who had previously experienced remissions following roentgen radiation (Cases 2, 12, 16, 23, 24, and 25). Improvement was observed in all members of this group after nitrogen mustard. The second group (Cases 5, 8, 10, 20 and 30) includes patients with widely disseminated Hodgkin's disease manifest by severe systemic intoxication but no demonstrable localized foci of activity. Following treatment with HN_2 remissions occurred and later recurrences of the disease were localized and responded readily to roentgen ray therapy. The third group consists of patients (Cases 3, 7, 9, 11, 14, 18, 22, and 31) who had received radiation over localized areas of activity without any beneficial effect and were considered to be roentgen ray resistant. These patients demonstrated significant clinical improvement after HN_2 therapy. The fourth group is represented by three patients (Cases 4, 6, and 19) who did not improve following roentgen therapy and who also failed to improve after nitrogen mustard. Subsequent radiation, however, produced remissions in these patients. In the first three groups mentioned clinical remissions were observed following a total of twenty-four courses (55 per cent) and in twenty patients (65 per cent). In summary, a total of twenty-three patients received benefit from nitrogen mustard therapy either through an immediate remission or, indirectly, by an apparent re-establishment of sensitivity to roentgen rays. Since three of the thirty-one patients in this series (Cases 13, 26, and 27) did not return for follow-up studies after discharge from the hospital, the twenty-three who obtained benefit following HN_2 therapy represent 82 per cent of the twenty-eight cases in which subsequent clinical and laboratory observations could be made. Five patients (Cases 1, 15, 17, 21, and 28) were not benefitted by HN_2 and subsequently expired.

In those patients in whom a remission was observed, the most troublesome symptoms of active Hodgkin's disease such as pain, pruritis, and occasional anorexia were relieved frequently after the third or fourth injection of the series. Fever was the most consistent clinical finding during exacerbations of Hodgkin's disease. In those cases in which a remission occurred, the temperature returned to normal during therapy or within a week after its completion. Many patients who noted no sustained subjective or objective evidence of regression of the disease nevertheless described a moderate degree of temporary relief from one or more of their symptoms. One patient (Case 14) developed a marked diffuse brown pigmentation of the skin during one exacerbation of the disease. Nitrogen mustard therapy was given and two weeks after completion of therapy the pigmentation had disappeared with the reappearance of a normal skin color. This decrease in abnormal skin pigmentation also was observed to a lesser degree in two other patients (Cases 2 and 9).

The toxic effects of the methyl-*bis* compound are fairly consistent. With the exception of one case in which diarrhea was present, anorexia, nausea, and vomiting were the only immediate toxic reactions. These symptoms occurred

during thirty-eight of the forty-four courses given and were usually apparent within one-half to three hours following the administration of therapy. In some instances nausea alone of short duration was described. In others, nausea and vomiting of twenty-four to forty-eight hours' duration were noted. Thrombophlebitis at the site of injection occurred during four courses. This was an annoying though not a serious complication.

Following the administration of twenty-three courses of nitrogen mustard therapy, thirteen patients developed maculopapular, markedly pruritic skin lesions over the trunk and extremities. The lesions appeared from one to six weeks after treatment. Morphologically the eruption first appeared as pink, maculopapular lesions, 2 to 5 mm. in diameter. They later became hemorrhagic in some cases. Throughout the development of the eruption and during its course, pruritis was marked. The lesions usually persisted for many weeks but were relatively few in number. In some cases the eruption was scattered diffusely over the body; in others it was confined to one or more extremities or to a single area of the trunk. Grossly the lesions were similar to those which occasionally are seen as skin manifestations of Hodgkin's disease. Microscopically, however, biopsy material obtained from three patients studied was described as vesiculobullous lesions presumably resulting from vascular damage. The walls of the surrounding vessels showed marked degeneration. In addition to the maculopapular lesions described, five of the thirty-one patients in this series developed the classic lesions of herpes zoster.

The most serious complication following nitrogen mustard therapy in this series resulted from bone marrow and lymphoid tissue destruction. Blood studies considered adequate and suitable for hematologic evaluation were done following thirty-five courses of therapy in twenty-six patients. A leucopenia below 2,000 white cells per cubic millimeter of blood was recorded following twenty-two courses. In fifteen the white blood count dropped below 1,000. The lymphocytes were the first cellular elements to reflect the toxic effect of the material. A decrease in the number of lymphocytes was observed as early as twenty-four hours after the first dose of nitrogen mustard in some cases. Before the institution of HN_2 therapy an absolute lymphopenia (below twenty per cent) was apparent in all of the patients studied. Twenty-five had less than 10 per cent and seventeen had less than 5 per cent lymphocytes before treatment. In the latter group (less than 5 per cent lymphocytes) a significant decrease in the number of circulating lymphocytes following therapy was obviously difficult to measure. Fourteen of the sixteen treatment courses given to patients whose blood contained more than 5 per cent lymphocytes before therapy demonstrated a significant drop in those cells within one to five days after the first dose was given. In twenty-three of the thirty-four courses subsequent blood studies revealed a return to the previous lymphocyte level or to a more nearly normal level. In nine courses, three weeks after therapy, 20 per cent of the circulating white blood cells were lymphocytes, a significant increase compared with the level before therapy. Four courses were followed by an immediate increase in lymphocytes. Two of these four experienced satisfactory remissions (Courses 17 and 42).

In addition to the lymphopenia present before therapy, in thirty-four of thirty-five instances an absolute monocytosis and an increased monocyte-lymphocyte ratio were found. The monocyte-lymphocyte ratio returned to normal and was accompanied by a satisfactory remission after fourteen of thirty-four courses. In eleven courses followed by no remission, the monocyte-lymphocyte ratio remained abnormal. When an alteration in the monocyte-lymphocyte ratio followed therapy, it consisted of both an increase in the total number of lymphocytes and a decrease in the number of monocytes. A direct correlation between variations in the monocyte-lymphocyte ratio and variation in the activity of the disease appeared to be present in twenty-five of thirty-four courses (74 per cent).

At the time when the leucopenia was most severe, approximately three weeks after therapy, all white blood cell elements were numerically reduced in equal proportion. Thrombocytopenia, when present, usually appeared a few days later than the leucopenia. A thrombocytopenia of less than 100,000 developed following seventeen of the thirty-four courses in which hematologic studies were made; thirteen patients had platelet counts below 50,000 and six below 10,000. Many of these patients developed petechiae; in one there was bleeding from the mucous membranes.

Twenty-four of the thirty-four treatment courses (71 per cent) followed hematologically demonstrated an increased reticulocyte count, as high as 10.8 per cent in one case, during exacerbations of the disease previous to nitrogen mustard therapy and during subsequent recrudescence of the disease. This reticulocytosis existed independent of the presence or absence of anemia. Following twenty-three of twenty-eight courses studied, the reticulocyte count dropped within two weeks following HN_2 therapy, to 0.0 in six instances; it remained above normal at its lowest point following nineteen courses (68 per cent). The most marked anemia was noted in most cases three to four weeks after HN_2 therapy and was recorded below 2.0 million red cells after eight of thirty-five treatment courses. The degree of anemia observed after therapy was dependent both on the red blood count before therapy and on the number of whole blood transfusions given the patient following therapy. A normal number of red blood cells present before treatment rarely was followed by a decrease of more than one to one and one-half million after nitrogen mustard. When the red blood cell level was low before therapy, a profound anemia frequently developed after HN_2. Whole blood transfusions were adequate for maintenance preceding marrow regeneration.

Serial sternal marrow studies were made in ten cases. Three of these revealed increases in reticulum cells, monocytes, and plasma cells before therapy. In five cases a left shift in the erythroid series was seen before treatment but there was no significant quantitative alteration in any of the normal cell types. All ten marrow samples studied demonstrated a moderate to marked hypoplasia of all cellular elements after treatment with HN_2. The diminution in marrow elements was apparent to some extent by the time therapy was completed five days after the first dose. Hypoplasia became more marked during

the subsequent two weeks and in most cases aplasia followed. Only fibroblasts and fat cells remained. After the period of aplasia, a normal regenerative process occurred with the appearance of young erythroid and myeloid forms in increasing numbers. Within five to six weeks after therapy, when the peripheral blood picture was approaching normal, a hyperplasia and left shift of erythroid and myeloid elements and megakaryocytes were seen.

Serial blood sedimentation rates were determined following thirteen courses. A direct relation between clinical benefit and improvement in sedimentation rate was observed in eight of thirteen patients studied (62 per cent.) The administration of folic acid in doses of 30 to 50 mg. daily during and after approximately one-half of the thirty-four courses observed did not seem to alter the cytotoxic effects of mustard therapy. Whole blood transfusions similarly did not alleviate the toxic depression of the marrow nor did they aid in maintaining a normal peripheral white blood cell or platelet level. All patients with a leucopenia below 2,000 cells were given penicillin until the white blood count rose above this level. In three cases there was secondary infection directly associated with the granulocytopenia; the infection was controlled by penicillin in each case.

Both C. E. (Case 12) and G. T. (Case 14), whose histories are summarized below, exhibited extensive damage of the hematopoietic system with return to a normal equilibrium in one and a partial return in the other. The latter patient (Case 14) experienced a longer and more complete remission than did the former (Case 12). However, in Courses 9, 12, and 14 (Cases 5, 7, and 9) illustrated in Table I, clinical remissions of two, five, and two months respectively were observed in patients who had only minimal evidence of toxic damage to the blood and lymphoid systems.

CASE 12.—C. E., a 35-year-old white woman, entered University Hospital during March, 1947 complaining of epigastric pain, weight loss, weakness, and cough. The illness began during the third trimester of the patient's last pregnancy during the fall of 1944 when she noticed a swelling in the left side of the neck. A few weeks later the skin became yellow and pruritic. About three months after the uncomplicated full-term delivery of a normal female child, the patient was referred to this clinic because of persisting cervical adenopathy. After a left supraclavicular lymph node biopsy in April, 1945, the diagnosis of Hodgkin's disease was made. The patient then received roentgen therapy over the right and left cervical regions and the mediastinum. During the fall of 1945 she received further radiation over the axillae, mediastinum, and epigastrium. She felt well until two months before admission when she developed epigastric pain which was more pronounced after eating. She stated that solid foods seemed "to get stuck" before reaching the stomach, causing pain which lasted more than an hour. These symptoms progressed and were accompanied by cough, weakness, and weight loss.

Physical examination revealed a pale, thin individual with cervical and axillary, nontender, firm, discrete nodes. Roentgen ray studies of the esophagus demonstrated an obstruction just behind the bifurcation of the trachea. A calcified inferior bifurcation node was visualized and roentgen ray findings suggested the possibility of ulceration into the esophagus. Following the administration of 25 mg. of *bis* (β-chloroethyl) amine the patient became nauseated and vomited. Six days later, roentgen ray examination revealed less evidence of obstruction and stenosis of the esophagus. For approximately two months the patient felt well and was able to eat solid foods without pain or difficulty. At that time dysphagia returned, became progressively more severe, and was accompanied by cough.

Physical examination revealed a widening of the mediastinum to the right. A second course of 22.5 mg. of nitrogen mustard was given. During the week after HN_2 therapy the patient noticed her cough becoming more severe with swallowing and reported that she had coughed up food particles. A diagnosis of esophageal-bronchial fistula was made and tubal suction via the esophagus was instituted. Fluoroscopic examination confirmed the diagnosis of fistula. The patient had a pruritic maculopapular eruption over the extremities and chest at this time.

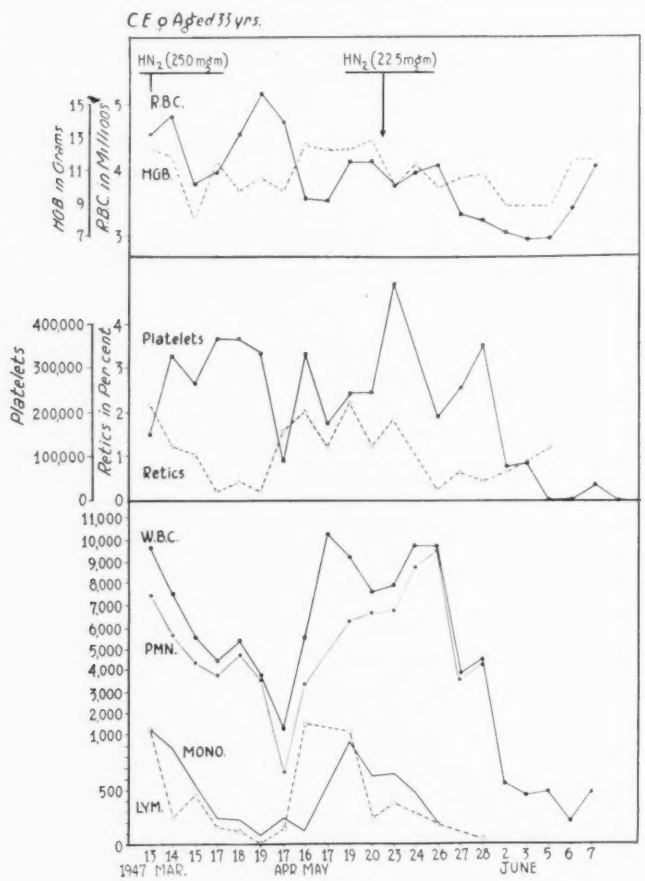


Fig. 1.

In spite of whole blood transfusions, glucose infusions, and penicillin, the condition of the patient worsened and she expired on the twenty-third hospital day, three months after the first course of nitrogen mustard. The skin eruption became more extensive and severe about one week after the second course of mustard therapy. The anatomic findings at autopsy were: (1) Bronchoesophageal fistula, (2) necrotic ulceration of the esophagus, (3) mediastinal lymphadenitis, (4) pulmonary infarction and emphysema, (5) fibrinous pleuritis, (6) multiple gastric ulcers with mucosal hemorrhage. The hematologic findings in this case are illustrated in Fig. 1.

CASE 14.—G. T., a 16-year-old white girl, was admitted to the University Hospital in February, 1947, complaining of weakness, enlarged glands, itching of the skin, and cough. In 1944 the patient noticed increasing weakness, easy fatigability, and enlarged left cervical

nodes. A diagnosis of Hodgkin's disease was made following study of a lymph node biopsy section and the patient was referred to this clinic. Following biopsy, the patient received multiple courses of roentgen therapy over the mediastinum, lungs, abdomen, and cervical and inguinal regions. The patient noticed no peripheral adenopathy during subsequent exacerbations but returned for roentgen ray treatment repeatedly because of recurrence of the weakness and weight loss. Six months before admission for HN_2 therapy the patient said she had severe generalized pruritis which continued until admission. One month before admission she developed a cough productive of thick yellow-white sputum. Pain in the substernal region accompanied the cough. On admission, fever, night sweats, occasional chills, productive cough, and weakness were described.

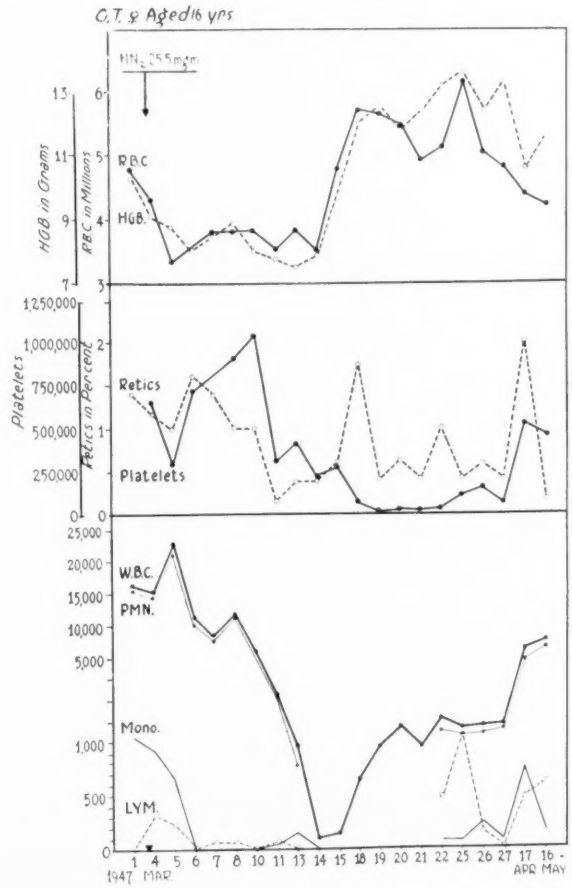
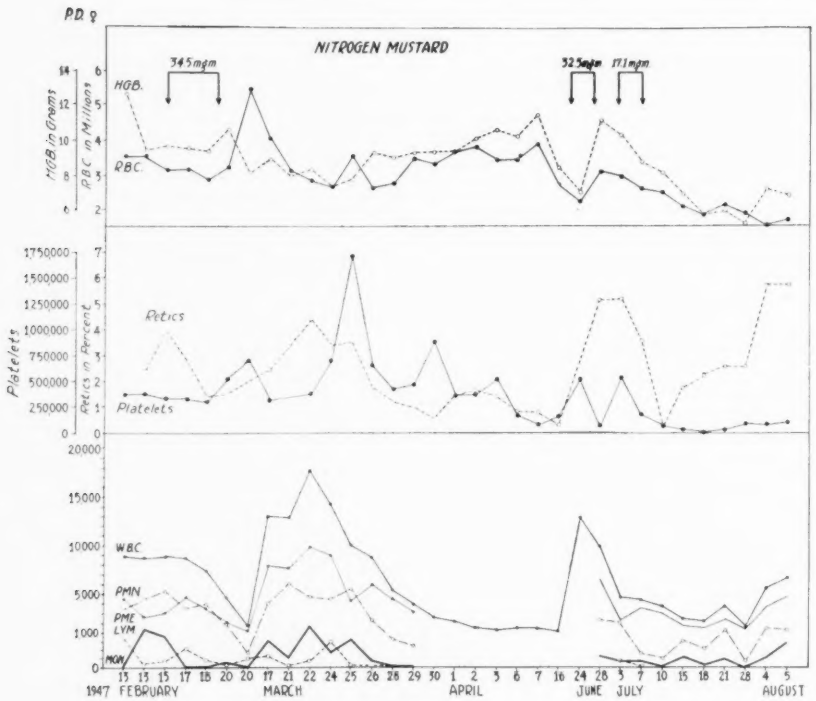


Fig. 2.

Emaciation, dark brown pigmentation of the skin, and a papular eruption over the entire body with excoriations were present. There was no significant adenopathy. Roentgen ray studies revealed a right pleural effusion with apparent involvement of the right upper lobe lung parenchyma by Hodgkin's process.

The patient received five daily doses of *bis* (β-chloroethyl) amine hydrochloride (25 mg.) with no untoward reaction. Ten days after the first dose roentgen studies revealed a diminution in the pleural effusion and increased aeration in the right lung apex. The temperature, 102 to 105° before therapy, was observed to be 105° on the first day of therapy. Two days after the first dose of nitrogen mustard the temperature was 100° and varied


P.D. ♀
NITROGEN MUSTARD
345 mgm
325 mgm
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HGB. in Grams
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Retics
Platelets
W.B.C.
PMN
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AUGUST

Fig. 3.

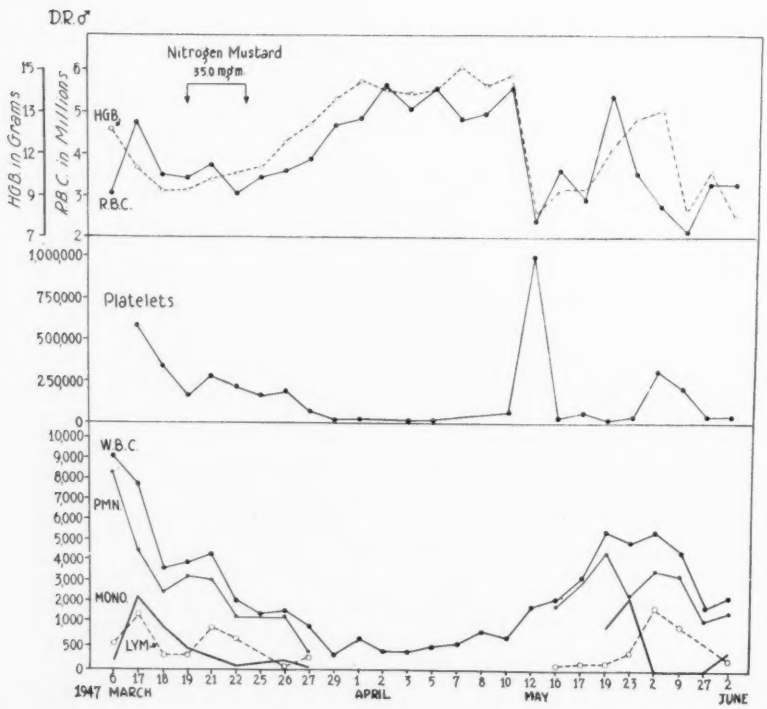
D.R. ♂
Nitrogen Mustard
350 mgm.
HGB.
R.B.C.
HGB. in Grams
R.B.C. in Millions
Platelets
W.B.C.
PMN.
MONO.
LYM.
1947 MARCH
APRIL
MAY
JUNE

Fig. 4.

between 98.0° and 101° until seventeen days after the beginning of therapy, at which time it returned to normal. The hematologic changes are illustrated in Fig. 2. The patient received 30,000 units of penicillin every three hours from the second day of treatment until discharge and 50 mg. folic acid orally every day after the twelfth day following the first dose of nitrogen mustard. She received four transfusions of 250 c.c. of whole blood during the second week after treatment. Pruritis and the papular eruption became less noticeable and were absent when the patient was discharged twenty-four days after the beginning of treatment. At the time of discharge the brown skin pigmentation had almost disappeared. Appetite, sleep, and general well-being were improved. The severity of the cough had greatly decreased and only a small amount of thin, clear sputum was obtained. Two weeks after discharge the patient returned feeling well except for a recurrence of the skin eruption with pruritis accompanied by lesions similar to those of herpes zoster. Although the herpetiform lesions disappeared in three to four weeks, the papular pruritic eruption persisted intermittently until a second course of nitrogen mustard was given six months later following a recurrence of fever and a progression of the pulmonary lesions.

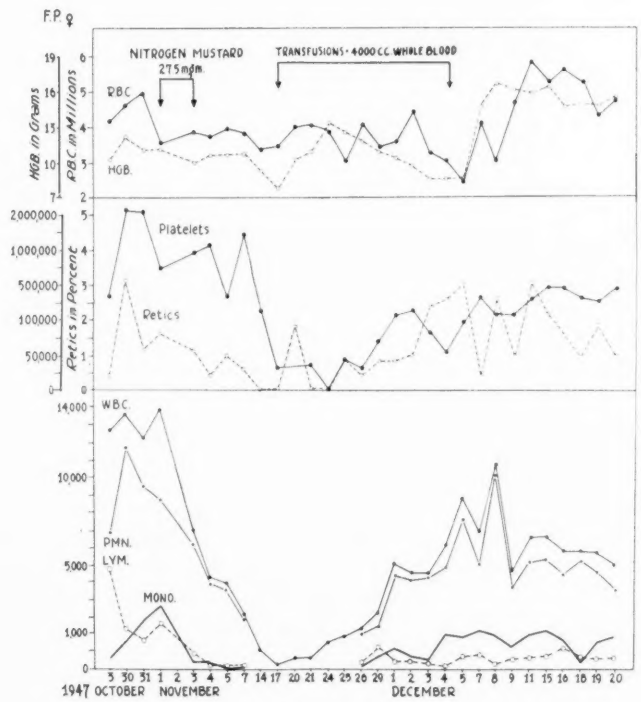

Fig. 5.

CASE 31.—F. P., a 29-year-old white married woman, was first diagnosed as having chronic lymphatic leucemia on the basis of a supraclavicular lymph node biopsy nine years before admission. For five years she received roentgen therapy over multiple areas of recurrent adenopathy. During most of that time she was able to maintain normal activity. Four years before admission to the hospital, a second lymph node biopsy gave a diagnosis of Hodgkin's disease and this diagnosis was reinforced by a review of the old slides. Since then the patient had continued to receive occasional roentgen ray treatments. During the six months preceding this admission she noted progressive weakness, dry cough, intermittent afternoon fever, substernal chest pain, and pain over the right inferior angle of the scapula. Roentgen ray studies revealed an enlargement of the mediastinal shadow which was inter-

preted as being due to adenopathy. The patient was given a total of 1,200 roentgens in air over each of three fields: anterior and posterior mediastinum, and right lateral chest. This latter therapy did not bring about relief of symptoms. On admission to the hospital the patient also had complained of a sense of fullness in the epigastrium and pain in the right upper quadrant for minutes to hours following the ingestion of any type of food.

Roentgen ray studies of the gastrointestinal tract, gall bladder, and kidneys demonstrated a distortion suggesting adenopathy in the right lumbar and celiac axis nodes. The gall bladder could not be visualized. Basal metabolic rate on admission was plus 3 per cent; blood urea nitrogen was 11.5. Prothrombin was 86.5 per cent, bromsulfalein, 10 per cent in both specimens, direct van den Bergh, 0.2, indirect, 0.75. Total protein was 6.75, albumin 3.75, and globulin 3.00. The serial hematologic studies are illustrated in Fig. 5.

The patient was given two doses of methyl-*bis* (β-chloroethyl) amine hydrochloride of 11.0 mg. on two successive days and 5.5 mg. on the third day. The patient experienced nausea and vomiting during and for one day after the course of therapy. The pain disappeared during the first week after treatment. A cholecystogram ten days after the first dose revealed a normally functioning gall bladder. The patient was discharged eleven days after the first dose of HN_2 apparently improved in every respect.

Six days after discharge the patient was readmitted, having felt well until three days after discharge. At this time she experienced fever, chills, malaise, nausea, and vomiting. Physical examination revealed an ulceration of the gingiva and petechiae on the soft palate. The abdomen was diffusely tender. The oral temperature was 102° F. The white blood count was 150 and the red blood count was 3.36 million; no platelets or reticulocytes were seen. The sternal marrow was aplastic; only fibroblasts, fat and plasma cells were found. The prothrombin was 44 per cent of normal, total protein 6.00 Gm. per cent, albumin 3.42 Gm. per cent, globulin 2.58 Gm. per cent. There was 40 mg. per cent albuminuria and a blood urea nitrogen of 31.5.

The patient was given 50,000 units of penicillin every three hours but continued to have a septic temperature. No new physical signs developed until five days after admission when a rapidly progressive jaundice appeared. At this time the van den Bergh was indirect 13.2, direct 10.35; cephalin was 1 plus, total protein 6.75, albumin 2.37, and globulin 3.38. Thymol turbidity was 20. During the course of this admission 3,500 c.c. of whole blood were given without untoward reaction. Streptomycin (4 Gm. daily) was started on the fourth hospital day. The patient became distended on the seventh hospital day and a Miller-Abbott tube was inserted. On the following day a right lower lobe pneumonia and atelectasis became manifest. Ten days after admission a mass was palpated in the right lower quadrant. A barium enema demonstrated a perforation of the posterior aspect of the cecum with escape of the barium into the retroperitoneal space. An incision and drainage of the retrocecal, retroperitoneal abscess were done. The temperature returned to normal within three days after surgery and the patient began to improve. *Escherichia coli* and staphylococci were obtained on culture of the abscess fluid.

The van den Bergh, blood protein, prothrombin, and lung findings returned to normal within one month after the abscess drainage and at that time the drainage tube was removed. Priodax roentgen ray studies revealed closure of the abscess cavity. The cecal perforation could not be demonstrated two weeks after surgical intervention.

DISCUSSION

Remissions of one to ten months have been observed following HN_2 therapy in the series of cases presented in this report. There appear to be several types of patient who may obtain benefit from this form of therapy. First, nitrogen mustard has produced remissions in the clinical course both of patients who had never been given roentgen therapy and in patients who had previously received beneficial effects from roentgen radiation. Second, HN_2 has been used

successfully in individuals who had widely disseminated Hodgkin's disease with severe systemic intoxication who were therefore unsuitable subjects for radiation therapy. Third, a regression of the disease after nitrogen mustard therapy has been observed in cases in which recent roentgen treatment over involved areas failed to produce beneficial results. Finally, an indirect benefit apparently was obtained in selected patients who previously had been considered roentgen ray resistant and who demonstrated no apparent improvement after nitrogen mustard therapy. In these patients an apparent resensitization to roentgen rays was established.

One of the most consistent clinical signs of an exacerbation of Hodgkin's disease in the present series was the presence of fever, either Pel-Ebstein or septic in type. As exemplified by G. T., Case 14, a decrease in temperature during or within a few days after therapy was observed sufficiently often to justify the use of this observation as an early index of the efficacy of the treatment. Relief of pain, observed in patients G. T. and F. P. (Cases 14 and 31), was also an early manifestation of a remission. Markedly pruritic skin lesions, an annoying counterpart of many cases of Hodgkin's disease, disappeared shortly after HN_2 therapy in G. T. (Case 14). Objective signs of a clinical remission are exemplified by the case report of G. T. In the presence of extensive pulmonary involvement, HN_2 therapy was followed by increased aeration of the lung, a diminished amount of sputum, and a decreased cough. Another objective change was observed in F. P. (Case 31) in whom the gall bladder, nonfunctioning before treatment, was reported to be normal after HN_2 therapy. The symptoms suggestive of gall bladder disease before therapy also disappeared. Skin pigmentation, a sign occasionally encountered in Hodgkin's disease, disappeared within a few days after HN_2 therapy in a few cases (Case 14). In Cases 14 and 31 a regression of the disease process was demonstrated in organs other than those usually associated with the lymphoid system. The toxic reactions to the nitrogen mustard therapy were fairly consistent, for example, nausea and vomiting occurred as an immediate toxic effect following each dose of HN_2 in 78 per cent of the courses given. The thrombophlebitis observed at the site of intravenous injection of this medication was an annoying but minor complication. The causal factor in the development of skin lesions in a number of patients after HN_2 therapy remains to be determined. It cannot be stated whether this was a cutaneous spread of Hodgkin's disease after nitrogen mustard or a toxic effect resulting from local or systemic vascular damage. The profound leucopenia and thrombocytopenia observed after HN_2 therapy reflects the extensive toxic damage to the hematopoietic system. The fact that the lymphocytes were the first cellular elements to be decreased in the peripheral blood suggests that the lymphocyte is the most rapidly destroyed of the formed elements of the blood. That the destruction of blood cell elements occurs as a result of a central toxic effect as well as a peripheral one is demonstrated by the concomitant destructive hypoplasia and aplasia of the bone marrow observed during the period when the peripheral count is lowest. The appearance of hyperplasia of the bone marrow and increased numbers of young

forms of myeloid and erythroid elements as well as megakaryocytes while the peripheral cellular elements are increasing during the recovery phase indicates the duration and the temporary nature of this toxic effect on the blood cell forming precursors of the hematopoietic system.

The most consistent hematologic abnormalities in Hodgkin's disease before therapy are lymphopenia, monocytosis, and reversed monocyte-lymphocyte ratio. The subsequent regeneration of the lymphoid tissues and return to normal of the circulating lymphocytes after therapy re-establishes a more nearly normal monocyte-lymphocyte ratio. In this series of patients the return of the monocyte-lymphocyte ratio toward normal was the most consistent laboratory finding associated with a clinical remission.

An exacerbation of Hodgkin's disease in these cases was also reflected to some extent by an absolute reticulocytosis before therapy regardless of the presence or absence of anemia. The alterations in the reticulocyte count accompanying the bone marrow changes after HN_2 therapy prohibit its use as a prognostic index of the results of treatment.

In considering patients for nitrogen mustard therapy, it should be noted that the danger of the toxic effect of HN_2 on the bone marrow is minimized by two factors. One of these is the ability of antibacterial chemotherapeutic agents to alleviate the danger of infection resulting from temporary agranulocytosis and the other is the tremendous capacity of hematopoietic tissue to regenerate despite extensive damage by HN_2. Similarly, evidences of bone marrow involvement by Hodgkin's disease before therapy with accompanying anemia, leucopenia, and thrombocytopenia do not constitute an absolute contraindication to HN_2.

The fact that no consistent relationship was observed in some cases following nitrogen mustard therapy between the appearance of a clinical remission and the amount of hematopoietic and lymphoid tissue damage as measured by peripheral blood and sternal marrow changes indicates that hematopoietic tissue damage is not necessarily a criterion of the benefit to the patient resulting from this treatment. Two patients (Cases 11 and 29) in whom a satisfactory regression of the disease was observed not only failed to present gross evidence of hematopoietic damage on study of the peripheral blood but actually were observed to have an immediate increase in circulating lymphocytes following therapy.

The case reports presented described some unusual complications which might be considered as toxic reactions following treatment because of their occurrence shortly after nitrogen mustard therapy. Treatment with HN_2 was followed in one case by a perforation of the esophagus and esophageal and gastric mucosal ulcerations, and in another by a cecal perforation; gastric and duodenal ulcers were observed in a third. One patient developed marked jaundice and evidences of severe liver damage shortly after treatment, findings which returned to normal a few weeks later. It cannot be determined whether any or all of these changes are the direct result of the action of nitrogen mustard or are a secondary manifestation of the destruction of active Hodgkin's

tissue. If the former is true, the destructive effect on the gastrointestinal mucosa of experimental animals by toxic doses of nitrogen mustards may also be observed in some cases at therapeutic dose levels in human beings.

Nitrogen mustard appears to be a valuable adjunct to the therapy of Hodgkin's disease although it does not replace roentgen therapy. A comparison of previously recorded results following roentgen radiation with those following the selective use of both roentgen radiation and nitrogen mustard therapy suggests that the combination of therapeutic agents may offer greater hope for increased life expectancy than either used alone.

SUMMARY

Thirty-one cases of Hodgkin's disease were treated with a total of forty-four courses of methyl-*bis* (β-chloroethyl) amine hydrochloride.

Beneficial results were observed in twenty patients receiving twenty-four courses. Indirectly, three other patients benefited through an apparent resensitization to roentgen rays. Improvement was characterized in most instances by an immediate disappearance of fever, itching, and pain. Brownish pigmentation of the skin was observed to decrease in several cases as did Hodgkin's skin lesions, splenomegaly, hepatomegaly, and adenopathy.

The toxic effects of HN_2 on hematopoietic and lymphoid tissues as reflected in peripheral blood and sternal marrow changes are described and discussed. These changes are contrasted with alterations in hematologic equilibria observed during exacerbations of Hodgkin's disease. Lymphopenia with a reversed monocyte-lymphocyte ratio is frequently observed during the active phase of Hodgkin's disease. A regeneration of lymphocytes and return of the monocyte-lymphocyte ratio toward normal is the most consistent laboratory finding associated with a clinical remission. An absolute reticulocytosis has been observed in many cases during the active phase of Hodgkin's disease before therapy regardless of the presence or absence of anemia. It is observed that no apparent relationship exists in selected cases between the amount of measurable damage to the hematopoietic and lymphoid tissues and the occurrence of clinical remissions. Bone marrow hypoplasia proceeding to aplasia and followed in every instance by complete regeneration to the previous level and in some cases to a more normal level within a few weeks after therapy was observed. Bone marrow involvement by the Hodgkin's disease process before therapy is not considered a contraindication to HN_2 therapy.

An immediate toxic reaction of nausea and vomiting was observed consistently after HN_2 therapy. Case reports have been presented describing unusual complications which followed HN_2 therapy. These include ulceration and perforation of the mucosa of the gastrointestinal tract. Skin lesions macroscopically similar to those frequently seen in Hodgkin's disease appeared in an unusually large number of cases following HN_2 therapy.

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THE EFFECT OF LIVER EXTRACT AND VITAMIN B_{12} ON THE MUCOUS MEMBRANE LESIONS OF MACROCYTIC ANEMIA

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FOR more than twenty years we have been studying the mucous membrane lesions of persons with macrocytic anemia and have observed that they usually are relieved by liver extract and Ventriculin. A great opportunity to study more carefully the pathogenesis of these lesions appeared with the advent of pteroylglutamic acid (folic acid) in 1945[1] and 5-methyl uracil (thymine) in 1946,[2] two pure chemical compounds which are effective in producing a hematologic response in certain types of macrocytic anemia.

As soon as it had been demonstrated* that synthetic folic acid and synthetic thymine were effective in producing a hemopoietic and clinical response in persons with pernicious anemia and related anemias,[1-4] additional studies on these substances were planned. These studies were directed toward answering the following questions: What clinical syndromes are affected by these chemical compounds? What is the relation of the structure of the molecule of these compounds to their antianemic properties? What are the relative clinical and hematologic effects of these substances as contrasted with each other and as contrasted with liver extract? How well do these substances maintain patients with pernicious anemia, nutritional macrocytic anemia, and tropical sprue? As a partial answer to this last query, we (and many others), during the past two and one-half years, have found that folic acid maintains the blood levels in persons with pernicious anemia just as well as liver extract but that it does not offer a complete treatment in most cases since it does not prevent or relieve subacute combined degeneration of the spinal cord.[5, 6] It appears from our studies that folic acid is preferable to liver extract in the maintenance of persons with nutritional macrocytic anemia and tropical sprue.[7] We have found that thymine, like folic acid, is not a complete treatment in most cases of pernicious anemia since it neither prevents nor relieves the subacute combined degeneration of the spinal cord, although it does maintain the blood levels very well.[8] Unpublished observations show that massive doses of thymine are as effective as liver extract or folic acid, both clinically and hematologically, in maintaining persons with nutritional macrocytic anemia and tropical sprue. The very large dose required, however, makes thymine impractical as a therapeutic agent.

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In our two and one-half years of study, the administration of either folic acid or thymine did not result in healing the very severe mucous membrane lesions in cases of pernicious anemia. In every instance the administration of liver extract was followed by prompt improvement. This improvement lasted for varying degrees of time after the cessation of liver extract therapy. The earliest relapse occurred one month after therapy was discontinued, and several patients had had no recurrences twelve months after therapy was discontinued. The great majority had a recurrence from within one to nine months after the last injection.

Three of the patients were uncooperative and, feeling very much better, would return to work and not wish treatment again. When they relapsed, they returned to us. These three patients had, at intervals, treatment with massive doses of thymine, parenteral liver extract, and oral administration of folic acid. In addition two of them had treatment with vitamin B_{12}, the newest vitamin to be isolated[9, 10] and found to be effective in producing a clinical and hematologic response in persons with addisonian pernicious anemia, nutritional macrocytic anemia, and tropical sprue.[11-13] The fiery redness and intense pain of the mucous membrane lesions were relieved, at least temporarily, in these two patients. A case history which is representative of the patients with severe lesions follows.

J. B., a 48-year-old white man. A diagnosis of pernicious anemia was first made in May, 1944. At that time he complained of general weakness, dyspnea and palpitation on exertion, soreness of mouth and tongue, swelling of feet and legs, and paresthesias of the extremities. The tongue was smooth (severe atrophy of papillae) and red, the gums were swollen and injected, and scars were present at the angles of the mouth. Sensory changes suggestive of peripheral neuritis were noted. The initial blood values were: red blood cell count, 1.25 million, hemoglobin, 5.1 Gm. (33 per cent), reticulocytes, 1.8 per cent, white blood cell count, 3,250, packed cell volume, 16, mean corpuscular volume, 128, mean corpuscular hemoglobin, 40.8, and mean corpuscular hemoglobin concentration, 31.8. Many megaloblasts were present in the aspirated sternal marrow. Other pertinent laboratory data were: achlorhydria and achylia after histamine stimulation, slightly elevated icteric index, and negative gastrointestinal x-ray studies. Therapy with a small amount of an experimental liver fraction resulted in partial relief clinically and hematologically.

Within a period of six months the anemia and associated symptoms relapsed. The symptoms and physical findings were essentially the same as noted on the first occasion. The blood counts were: red blood cells, 1.39 million, hemoglobin 4.4 Gm., and reticulocytes 0.2 per cent. Therapy with commercial liver extract (Reticulogen) induced a remission rapidly. A peak reticulocyte count of 37.3 per cent was obtained on the sixth day of therapy. Fifty-seven days after therapy was started the red blood cell count was 5.08 million and hemoglobin was 13.1 grams. Clinical improvement was just as remarkable. The signs and symptoms of glossitis and stomatitis subsided rapidly, and regrowth of lingual papillae was observed.

After an interval of nine months without therapy the patient was observed a third time in severe relapse in September, 1945. In addition to the symptoms and physical signs of anemia per se, there were severe glossitis and stomatitis and moderately severe paresthesias and physical signs of peripheral neuritis. The initial red blood cell count was 1.64 million and hemoglobin was 7.1 grams. The patient was given 100 mg. of folic acid daily by mouth for twenty days. A peak reticulocyte count of 19.2 per cent was obtained on the fifth day of therapy. Sixty-five days after folic acid was started the red blood cell count was 4.97 million and hemoglobin was 12.5 grams. Symptoms and physical signs of glossitis and stomatitis subsided entirely within ten days, and on the patient's release from the hospital the neurological examination was negative.

A fourth relapse developed within five months after the folic acid therapy was discontinued. The symptoms and physical findings in March, 1946, were essentially the same as noted on the three previous occasions, except that there was evidence of progression of the peripheral neuritis. The blood counts were: red blood cells, 1.55 million, hemoglobin, 6.2 Gm. (40 per cent), white blood cells, 3,050, reticulocytes, 0.3 per cent, packed cell volume, 20, mean corpuscular volume, 129, mean corpuscular hemoglobin, 40, and mean corpuscular hemoglobin concentration, 31. Therapy consisted of 5-methyl uracil (thymine), 6 Gm. daily by mouth for nineteen days. A peak reticulocyte count of 16 per cent was obtained on the eleventh day of therapy. Twenty-one days after therapy was started the red blood cell count was 2.39 million and hemoglobin was 8.9 Gm., an increase of about one-half million red blood cells and 3.1 Gm. of hemoglobin in three weeks' time. Although studies of sternal marrow which was aspirated just after the peak of reticulocytosis revealed a normoblastic reactive stage comparable to that observed during therapy with liver extract and, on another occasion, with folic acid, the anemia began to relapse soon after therapy was discontinued. Neither the stomatitis and glossitis nor the nervous symptoms were relieved. While the patient was taking thymine the glossitis and stomatitis became worse, with swelling and increased burning soreness of mouth and tongue and ulceration and fissuring of mucosa of lower lip.

The patient left the hospital against advice, thus interrupting therapy. However, on June 28, 1946, he returned to the clinic and was started on folic acid, 10 mg. daily by mouth. At that time symptoms and signs of glossitis and stomatitis were still present. The patient received the folic acid (10 mg. four times a day) for thirteen days. A peak reticulocyte count of 26.6 per cent was obtained on the eleventh day of therapy. During the twenty-three days he was observed, the red blood cell count increased from 0.91 million to 2.19 million, and hemoglobin from 4.0 to 7.9 grams. Throughout that period glossitis and stomatitis persisted. The patient insisted on leaving the hospital again.

He was readmitted to the hospital in September, 1946. Despite the persistence of severe anemia and glossitis and stomatitis, he had worked regularly at a cotton mill during the previous sixty days. During that time, however, paresthesias recurred and became progressively worse, and disturbed locomotion developed. The patient was given liver extract intramuscularly and improved gradually both clinically and hematologically. On December 18, 1946, the red blood cell count was 4.60 million and hemoglobin was 12.7 grams. The patient refused more therapy and returned to work.

During June, 1947, the patient had an insidious onset of soreness, stinging, and burning sensation of the mouth and tongue, progressive general weakness and dyspnea on exertion, and mild paresthesias of extremities. Six weeks later, in July, 1947, when he returned to the outpatient clinic, a moderately severe glossitis and stomatitis, anemia, chronic peripheral neuritis, and possibly posterior column degeneration were found. Blood counts were: red blood cells, 2.00 million, hemoglobin, 8.0 Gm. (52 per cent), reticulocytes, 0.8 per cent, white blood cells, 3,200, packed cell volume, 26, mean corpuscular volume, 130, mean corpuscular hemoglobin, 40, and mean corpuscular hemoglobin concentration, 31. A single dose, 1 c.c., of a highly refined commercial liver extract was given intramuscularly. During the subsequent ten days the stomatitis, glossitis, and cheilosis cleared rapidly, and regeneration of lingual papillae began. The anemia likewise improved; the blood values obtained four weeks later were: red blood cell count, 3.86 million, hemoglobin, 12.2 Gm. (79 per cent), reticulocytes, 0.4 per cent, and white blood cell count, 6,550. The patient again refused more treatment and returned to work.

During October, however, there was a gradual return of all symptoms which by the time of admission to the hospital a month later had become severe. The patient's tongue was swollen; deep dental impressions were present at the tip; all surfaces were red and the papillae apparently were severely atrophied; the buccal mucosa appeared slightly swollen and large areas were hyperemic, particularly opposite the line of closure of the teeth; tooth indentations marked the mucosa of the lower lip, and several oozing fissures extended laterally externally from the left angle of the mouth.

Folic acid, 10 mg. daily by mouth, was started on Dec. 14, 1947, and continued to March 3, 1948—a total of eighty days. It was then increased to 40 mg. a day for thirty-

four days, and finally to 50 mg. a day for an additional period of twenty-two days. After the dose of folic acid was increased to 50 mg. a day, the patient received simultaneously 600 mg. of niacinamide a day for seven days. Then during the next fifteen days the following vitamins (in addition to folic acid), with daily doses as indicated, were given: Vitamin A, 75,000 U.S.P. units; vitamin D, 3,000 U.S.P. units; thiamine 30 mg.; riboflavin, 15 mg., niacinamide, 450 mg.; ascorbic acid, 450 milligrams. While the patient was on this therapy there was considerable, but suboptimal, improvement of the anemia, but no appreciable beneficial effect, either from the folic acid alone or from the other vitamins, on the glossitis, stomatitis, and cheilosis resulted. The blood values at the beginning of therapy were: red blood cell count, 2.15 million, hemoglobin, 8.1 Gm. (53 per cent), reticulocytes, 0.6 per cent, and white blood cell count, 4,900. At the conclusion of therapy (a total of 137 days) they were: red blood cell count, 4.15 million, hemoglobin, 14.2 Gm. (92 per cent), reticulocytes, 2.2 per cent, and white blood cell count, 9,250. A peak reticulocyte count of 18.1 per cent was obtained on the eleventh day of folic acid therapy (10 mg. four times a day).

Despite the fact that the patient's blood values were as high as we had ever seen them, the stomatitis and glossitis were worse. The fiery redness was more intense. The pain made it almost unbearable, so on April 30, 15μg of vitamin B_{12} were given intramuscularly. Within twenty-four hours there were equivocal signs of improvement. After forty-eight hours there was definite fading of the hyperemia of the tongue and buccal mucosa. By the fifth day after the injection, the color of the mucosa of the tongue and oral cavity appeared about normal except in the most swollen areas of the lower lip; at this time the external fissures were dry and appeared to be healing. The tongue appeared less swollen, and by the thirteenth day many fine papillae were visible over the entire upper surface and the external fissures appeared completely healed. All soreness and burning sensation of the mouth, tongue, and external fissures began to subside within forty-eight hours after the injection and completely disappeared within five days.

SUMMARY AND CONCLUSIONS

These studies show that certain patients with addisonian pernicious anemia have severe mucous membrane lesions that are not relieved by the administration of massive doses of thymine or large doses of folic acid. These severe mucous membrane lesions are characterized by a fiery red appearance and, in most instances, excruciating pain to the patients. These severe lesions have been seen only in patients with pernicious anemia, that is to say, patients who had gastric achlorhydria and achylia, and in each instance these lesions have occurred only in people who had subacute combined degeneration of the spinal cord. There seems to be a close clinical association between the gastric defect, the severe mucous membrane lesions, and the degeneration of the posterior and lateral columns of the spinal cord in these particular patients.

Without exception, the patients in our experience, as illustrated by the case history, have benefited from liver extract injections, and two of them were given vitamin B_{12} with similar relief for at least two weeks' time. These findings support our previous contention that neither thymine nor folic acid is a complete treatment for persons with pernicious anemia, whereas, in contrast, parenteral liver extract is.

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THE EFFECT OF 2, 3-DITHIOPROPANOL (BAL) ON GOLD TOXICITY IN RATS

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GOLD salts have been used in the treatment of rheumatoid arthritis since they were introduced by Forestier[1] in 1929. Toxicity sometimes occurring with the use of gold salts has been an important factor limiting the more general use of this form of therapy.

Treatment of toxicity due to gold for the most part has been unsuccessful. 2,3-Dithiopropanol (BAL) has been reported[3] by a group of investigators† to be successful in counteracting arsenic and mercury poisoning. These investigators made no report on the effect of BAL against gold toxicity.

Because of the value of BAL in the treatment of intoxication from other heavy metals, it seemed appropriate to study the effect of BAL in the prevention and treatment of toxicity due to gold. Clinical investigations will be reviewed separately; it is the purpose of this paper to report the effect of BAL in prophylaxis and therapy of the experimental gold toxicity in albino rats.

EXPERIMENTAL

Albino rats averaging 160 grams in weight were used as experimental animals. Gold sodium thiosulfate, $Na_3\,Au(S_2\,O_3)_2$, in a 37½ per cent aqueous solution was the gold salt used in every instance. BAL always was injected in doses of 0.0375 c.c., which is ten times the average dose recommended for the treatment of acute arsenic or mercury poisoning in human beings.‡ All injections of gold salt and BAL were made intramuscularly. The experimental animals were divided into three groups of twelve. Each of the rats in Group A was injected daily for seven days with an amount of gold sodium thiosulfate containing 1.8 mg. of gold. An injection of BAL was made into six rats of this group on the fifth and sixth experimental days. Each rat in Group B received 4.7 mg. of gold daily for four days. On the third and fourth experimental days, BAL was injected twice daily into half of the animals in this group. Each rat in Group C received 3.8 mg. of gold daily for seven days. BAL was injected into six of these animals twice daily on the first three days of the experiment and once daily on the fourth through the seventh day. At the end of each experiment the animals were killed and the organs were carefully examined.

RESULTS

All rats in Group A showed no change in general appearance, but they were less active and had diminished appetites. In behavior no differences were noted between the animals which received BAL and those that did not. One rat in the control group died on the third day; all others were killed on the seventh experimental day. Sections of the kidneys of the rats treated with BAL, in

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‡The distributors of BAL (Hynson, Westcott & Dunning, Inc., Baltimore, Md.) recommend injections of 2.5 mg. per kilogram body weight to be given several times daily for several days.

TABLE I. CONDENSED RESULTS OF EXPERIMENT

RATS	GOLD INJECTED	BAL INJECTED	KIDNEY PATHOLOGY	REMARKS
Group A (1) 6	1.8 mg. daily for 7 days	0.0375 c.c. twice daily for 2 days starting on fifth day	Moderate tubular degeneration with deposits of gold in tubules	Movements moderately slowed; rats killed on seventh day of experiment
(2) 6 (control)	1.8 mg. daily for 7 days	None	Same	Same
Group B (1) 6	4.7 mg. daily for 4 days	0.0375 c.c. twice daily for 2 days starting on third day	Severe tubular degeneration; extensive deposit of gold in tubules	Four rats died of toxic effects of gold before BAL was administered; two rats from B_2 transferred to B_1 as substitutes; four rats died at end of third day; all rats were listless, had poor appetites, ruffled fur, and pale eyes and tails
(2) 6 (control)	4.7 mg. daily for 4 days	None	Same	Four remaining control rats were listless, had poor appetite and pale eyes and tails; rats killed at end of fourth day
Group C (1) 6	3.8 mg. daily for 7 days	Starting on first day, 0.037 c.c. twice daily for 3 days then once daily for 4 days	Only moderate tubular degeneration; no deposits of gold salts in tubules	Rats appeared well; good appetite, normal fur, no pallor; animals killed on twenty-second day for examination
(2) 6 (control)	3.8 mg. daily for 7 days	None	Severe tubular degeneration with deposits of gold in tubules	Rats listless, inactive, had poor appetite, and were pale from second day; two rats died on second day, 2 on third day, and 1 on fifth day; remaining rat killed on eighth day for examination

Group A, revealed quite normal glomeruli; most tubules appeared to be entirely normal. Some tubular cells showed a moderate degree of granular degeneration; in the lumina of these tubules there was frequently an albuminous precipitate. The most striking abnormality was the pigmented precipitate found in tubular cells. Dark-orange and fine, red granules were seen in the epithelium of many convoluted tubules. There were some scattered deposits of golden yellow granules but these were relatively scant as compared with the fine orange-red deposits. Marked congestion of the interlobular vessels was present. The collective tubules were normal, but moderate congestion of the medullary capillaries was present. The kidneys of the control rats of Group A (those animals that received no BAL) showed the same changes in the same degree as were found in the rats treated with BAL (Table I).

Of Group B, four rats died before BAL was started on the third day. The experiment was continued with four rats receiving gold and BAL, and four rats served as controls. When BAL was started the entire group were listless, had ruffled fur, did not eat, and had little color in ears and tails. The rats injected with BAL died within ninety-six hours and the rats in the control group were killed on the fourth day.

In the kidneys of all animals in Group B there was severe degeneration of the epithelium in the convoluted tubules, in a patchy distribution in the epithelium and lumen. In the kidneys of rats that received no BAL, many convoluted tubules contained clumps of golden yellow granular pigment of various sizes. This pigment was found both in the epithelium and in the lumen. Some of the epithelium was necrotic and was fused to form a pale, granular, homogenous coagulum filling and sometimes blocking the lumen. Numerous eosinophilic casts were present in the collecting tubules and in the loops of Henle. Deeply stained eosin casts blocked some collecting tubules. Among those rats that were not injected with BAL, the collecting tubules of the kidneys contained pigment also; this was usually in small, light-golden granules.

In Group C, the six rats receiving gold and BAL simultaneously appeared to be entirely normal; they ate well and were normally active during the whole experiment. They were killed on the twenty-second day in order to examine the kidneys. The control rats (which received no BAL) were listless and inactive from the second experimental day onward. Two rats of this group died at the end of the second day, two died on the third day, and one rat died on the fifth day of the experiment. The one remaining rat of this group was killed on the eighth day.

Examination of the kidneys of rats from Group C showed interesting changes. A kidney from the rat which received no BAL weighed 1.55 grams as contrasted to 1.0 gram, the average weight of one kidney from rats in this group which were injected with BAL. In the kidneys of the rats that received gold and BAL there was only moderate degeneration of epithelium in the convoluted tubules. Some of the tubules contained a small amount of albumin and occasional casts. No metallic precipitate was observed in the tissue. The kidneys of the rats in Group C that received no BAL showed severe degenerative changes, necrosis, and gold precipitate in abundance exactly as seen in the rats of Group B that received no BAL.

In none of the experimental animals was there any evidence of abscess formation in the muscle into which injections of BAL or of gold sodium thiosulfate were made.

DISCUSSION

In order to be certain that the abnormalities seen in the kidneys of the experimental animals were produced by the gold sodium thiosulfate injected, the kidneys of two normal, untreated rats were carefully examined. They contained no orange-red or golden yellow precipitate. Parenchymatous elements were well preserved. Some tubules showed slight vacuolar degeneration of the epithelium, and occasional hyalin casts were seen. There was considerable congestion of the glomerular and interstitial capillaries. The pigment precipitated in the kidney tissue and the renal cellular damage observed in the rats of this investigation were exactly like the changes due to gold toxicity produced in earlier studies conducted by one of us.[4]

In these investigations the toxicity of gold was similar to that previously described in white rats.[4] The chief pathologic change was in the kidneys where

nephrosis similar to that caused by some other heavy metals resulted. When the dose of gold is sufficiently large, death may result quickly, as was the case in the animals in Group B. Because of this, the dose of gold was reduced in the studies conducted on the animals of Group C to permit sufficient time for any beneficial effects of BAL to be accomplished.

It is clear from these studies that even in large doses BAL had no therapeutic value when it was injected after extensive cellular damage already had occurred. When given simultaneously with large doses of gold sufficient to cause death in many animals of Group C, BAL proved to be effective in combating toxicity from gold. Even though some renal damage then occurred, it was of small amount and insufficient to cause systemic toxicity in the rats which lived and appeared well until they were killed in order to examine the tissues.

Besides the definite decrease in cellular damage, a conspicuous difference in appearance of the kidneys of the rats in Group C which were treated with BAL was that no precipitate of gold or gold salt was observed in the convoluted tubules. This observation suggests that BAL combines with the gold instead of the tissue cells and keeps it in the body in a nontoxic state or eliminates it as such so as to prevent any significant cellular damage or its precipitation in renal tissue. It appears that BAL successfully competes with the host tissues for combination with circulating gold. If BAL is present in sufficient amounts before gold becomes combined with or deposited in tissues, extensive cellular damage is prevented and life is sustained. The time element appears to be the most important factor in this competition for gold; injections of BAL made soon after poisoning are effective; the same amount of BAL given later in the course of gold administration does not protect against lethal toxicity.

It appears clear therefore that to be most beneficial in human beings in combating toxicity resulting from therapeutic injections of gold salts, BAL should be administered promptly when signs of toxicity are first observed; the longer the delay in use of BAL the less beneficial it likely will be. This is borne out in the clinical reports concerning BAL[5-7] and in unpublished clinical observations of the authors. Ragan and Boots[5] reported BAL to be effective in protecting rats against toxicity of gold. It is impossible to compare our results with theirs for no details of dosage or time relations were published in their report.

These animal studies suggest that the manner in which BAL acts to prevent toxicity due to gold is in all respects similar to its action against arsenic and mercury intoxication.[3]

SUMMARY

The toxicity from gold salts injected into albino rats is described. BAL had no protective effect when injected several days after toxic doses of gold were administered. When injected simultaneously with the administration of toxic doses of gold sufficient to be lethal to rats, BAL prevented important toxicity; the animals lived in apparently good health. These observations indicate that BAL is an effective antidote for gold if it is given in adequate amounts sufficiently early after the administration of gold salts. These results

further suggest that to have maximal effect in human beings who have toxicity from gold salts, BAL should be given as soon as signs of toxicity are observed.

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THE EFFECT OF FEEDING PROPYLTHIOURACIL AND CHOLESTEROL ON THE BLOOD CHOLESTEROL AND ARTERIAL INTIMA IN THE RAT

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ATTEMPTS to produce arteriosclerosis in the rat have uniformly met with failure. Cholesterol feeding per se has been attempted by many, without appreciably affecting either the blood cholesterol levels or the arterial intima.[1]

The nature of this resistance to the development of experimental atherosclerosis is unknown, and its elucidation is vital to the solution of the pathogenesis of the disease. That one of the factors involved in the process of resistance may be hormonal is suggested by the recent work of Steiner and Kendall[2] who found that thiouracil and cholesterol fed to dogs will produce atherosclerosis, while either agent when fed alone will fail to do so. This work is highly significant because the dog does not normally develop atherosclerosis and the experimental variety of the disease has never previously been induced in this animal. Previous work has shown that the thyroid gland is involved in the entire process of experimental atherosclerosis. In rabbits thyroidectomy renders the animals far more susceptible to the cholesterolemia and atherosclerosis which follow the feeding of cholesterol.[4] On the other hand, feeding desiccated thyroid or potassium iodide seems to protect the animals against the development of cholesterol-induced lesions and lowers the blood cholesterol levels.[3] We have been able to confirm the protective action of desiccated thyroid in another species, the chicken.[5] We also have observed the synergistic effect of cholesterol and thiouracil on raising the blood cholesterol levels of the chicken.[6] With these considerations in mind, we decided to use the antithyroid drug thiouracil in the rat in an attempt to break down the resistance of this species to the induction of atherosclerosis by cholesterol feeding.

PROCEDURE

White rats of the Harlan strain (originally Wistar) were used. They weighed approximately 100 to 150 grams at the start of the experiment. The rats were divided into six separate groups. Group 1 was maintained on a diet of dog biscuits‡ and water ad libitum. Group 2 received 5 per cent cholesterol suspended in cottonseed oil mixed with ground meal,‡ and tap water. Group 3 received 10 per cent cholesterol mixed with the basal diet. Groups 4, 5, and 6 received propylthiouracil§ either alone or mixed with cholesterol in

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the basal diet. The dose of propylthiouracil employed was 0.2 per cent at the start; it was increased to 0.3 per cent after fifteen weeks, and to 0.4 per cent after an additional five weeks. Propylthiouracil was dissolved and suspended in the drinking water in a concentration of 0.1 per cent. Group 4, consisting of eight rats, received the basal diet plus propylthiouracil in the dosage outlined. Group 5, consisting of twelve rats, received the basal diet plus propylthiouracil and 5 per cent cholesterol in oil. Group 6, consisting of eight rats, received the basal diet plus propylthiouracil and 10 per cent cholesterol in oil. In order to increase the food intake and to prevent scattering, milk powder and water were used as a binder and the feed was prepared in cake form. The amount of milk powder utilized amounted to approximately 20 per cent by weight of the total feed and added substantially to the protein and caloric value of the feed. The animals were weighed at intervals of three weeks and the blood cholesterol levels determined at that time. Two animals were chosen at random from Group 1 for cholesterol determinations and four animals from each of the other groups with the exception of Group 5, from which six animals were chosen at random for the cholesterol determinations. Blood was obtained from the tail vein, and the Schoenheimer-Sperry technique[7] was used for total cholesterol determination. Food and water consumption data were collected in each group at intervals of three weeks throughout the experiment. All animals were autopsied and all organs examined. Special attention was paid to the heart and aorta which were removed en bloc, slit open with fine scissors, and carefully examined for evidence of atheroma. Hematoxylin and eosin stained sections were made only of those aortas which appeared to show gross evidence of atheroma.

RESULTS

Food Intake.—Data in Table I indicate that the normal rat consumed 24 to 42 Gm. of food in twenty-four hours. The water consumption during the same period was 27 to 42 c.c. per rat. The food consumption of the 5 and 10 per cent cholesterol fed rats approximately equalled that of the controls, with intakes of 20 to 39 and 20 to 47 Gm. per rat per day respectively. The fluid intake for these rats was somewhat lower than for the control group, with a more marked discrepancy in the 10 per cent cholesterol fed group. The animals receiving propylthiouracil, either alone or in combination with cholesterol, had a lower food intake than either the normal controls or the cholesterol controls. The intake for the propylthiouracil group ranged from 20 to 28 Gm. per rat per day of food and 11 to 30 c.c. of water. The intake of the 5 per cent cholesterol-propylthiouracil group was 14 to 21 Gm. per rat per day and 14 to 30 c.c. per rat per day, and of the 10 per cent cholesterol-propylthiouracil group, 16 to 19 Gm. per rat per day and 13 to 20 c.c. per rat per day. It is apparent that the intake of food and water in the two latter groups was approximately half that of the control groups.

Body Weight.—The data are shown in Table II. At the commencement of the experiment, the animals weighed between 111 and 153 grams, with a

TABLE I. FOOD AND WATER CONSUMED PER RAT DURING A TWENTY-FOUR HOUR TEST PERIOD

GROUP	5/1/47		5/23/47		6/14/47		7/3/47		8/6/47		9/3/47		10/25/47	
	FOOD (GM.)	WATER (C.C.)	FOOD (GM.)	WATER (C.C.)	FOOD (GM.)	WATER (C.C.)	FOOD (GM.)	WATER (C.C.)	FOOD (GM.)	WATER (C.C.)	FOOD (GM.)	WATER (C.C.)	FOOD (GM.)	WATER (C.C.)
Normal	24	32	29	35	31	27	26	29	27	43	42	23	37	38
5 per cent cholesterol	20	24	28	24	23	20	34	32	26	39	32	29	49	19
10 per cent cholesterol	20	18	25	22	26	20	39	24	22	39	47	18	31	27
Propylthiouracil	20	21	24	19	24	20	28	11	12	30	23	15	26	13
5 per cent cholesterol-propylthiouracil	20	30	20	22	16	21	21	26	14	19	20	14	36	31
10 per cent cholesterol-propylthiouracil	18	15	15	16	16	20	19	15	16	15	16	13	30	21

TABLE II. AVERAGE BODY WEIGHT OF DIFFERENT GROUPS IN GRAMS

GROUP	NUMBER OF WEEKS ON DIET								
	0	2	6	10	14	20	24	28	38
Normal	111	178	223	250	278	316	288	317	328
5 per cent cholesterol	142	147	221	266	314	331	329	352	404
10 per cent cholesterol	135	155	239	282	312	279	345	364	425
Propylthiouracil	153	151	191	199	215	224	196	243	257
5 per cent cholesterol-propylthiouracil	147	138	191	188	189	185	158	220	160*
10 per cent cholesterol-propylthiouracil	145	163	170	169	165	150	156	225	168†

*Five animals only.
†Four animals only.

mean weight of 139 grams. The normal control animals gained steadily in weight and had reached a peak weight of 328 grams at the conclusion of the experiment. The 5 per cent cholesterol animals gained steadily in weight and were somewhat heavier than the normal controls, weighing 404 grams. The groups which received propylthiouracil either alone or in combination with cholesterol were far lighter than any of the control groups. Thus the propylthiouracil group weighed 257 grams at the conclusion of the experiment, and the 5 per cent and 10 per cent cholesterol-propylthiouracil groups 160 and 168 grams respectively. This correlates well with the figures obtained for food intake.

Blood Cholesterol Levels.—The cholesterol values for the normal controls showed no significant changes throughout the course of the experiment. The highest and lowest average values recorded were 53.5 and 95 mg. per cent respectively with a mean of 74.1 mg. per cent. There was no trend toward an increase of the blood cholesterol levels with increasing age. The 5 per cent cholesterol fed group showed a slight rise in the blood cholesterol level by the fourth week of feeding. By the eighth week of feeding the blood cholesterol level had risen to an average value of 136 mg. per cent and this was the highest average value attained by this group. Thereafter, it fell off gradually and fluctuated, but remained consistently higher than that of the controls. The blood cholesterol levels for the 10 per cent cholesterol fed group showed a rise parallel to and slightly greater than those in the 5 per cent cholesterol fed group. The highest average value for this group was 142 mg. per cent and was achieved by the eighth week of feeding.

In the propylthiouracil control group the blood cholesterol levels rose to 82.4 mg. per cent after four weeks of feeding, and subsequently rose to 167 mg. per cent by the eighth week. Thereafter there was a fluctuating decline, with the values in this group somewhat higher than those for the 10 per cent cholesterol fed group.

The 5 per cent cholesterol-propylthiouracil group showed an early rise of blood cholesterol levels to 97.2 mg. per cent after four weeks of feeding, and then a progressive rise to a high average value of 373 mg. per cent after sixteen to twenty weeks of feeding. Thereafter there was a gradual decline in the blood cholesterol levels. The highest blood cholesterol level in any individual in this group was 616 mg. per cent and was achieved after twenty-four

TABLE III. AVERAGE BLOOD CHOLESTEROL LEVELS OF DIFFERENT GROUPS IN MILLIGRAMS PER CENT

GROUP	NUMBER OF WEEKS ON DIET								
	0	4	8	12	17	22	27	31	38
Normal	54*	54	81	66	95	80	88	65	64
5 per cent cholesterol		77	136	102	107	113	95	81	101
10 per cent cholesterol		86	142	95	130	129	113	118	92
Propylthiouracil		82	167	104	139	138	94	130	63
5 per cent cholesterol-propylthiouracil		97	200	250	373	315	152	158	70
10 per cent cholesterol-propylthiouracil		155	254	283	345	303	114	288	79

*Average control value for all groups.

weeks on the diet. The 10 per cent cholesterol-propylthiouracil group showed the greatest early rise in the blood cholesterol of any of the groups, with a level of 155 mg. per cent after four weeks of feeding. This was almost three times the average control level. The blood cholesterol level rose steadily thereafter to 345 mg. per cent after sixteen to twenty weeks of feeding and then declined gradually.

In summary then, the feeding of either cholesterol or propylthiouracil alone in the dosage employed resulted in each case in a slight rise of the blood cholesterol levels above the normal. Five and ten per cent cholesterol in the diet produced rises in the blood cholesterol levels which were similar when the entire time course of the experiment is surveyed. When the cholesterol and propylthiouracil were combined in the diet, the rise in blood cholesterol was significantly greater than in the preceding groups. The combination of 10 per cent cholesterol with propylthiouracil gave somewhat higher levels during the first twelve weeks of the experiment than the 5 per cent cholesterol combined with propylthiouracil. The high average values on *all* diets were achieved before the twenty-second week of feeding and tended to decline thereafter. This correlates with a tendency on the part of the animals to failure of further weight gain, decreased food intake, and generally poor health which became apparent at this time.* Toward the end of the experiment it was necessary to substitute ordinary Friskies for the prepared feed for a few days at a time because of the sickly appearance of the rats. Propylthiouracil alone produced a cholesterolemia equal to that produced by cholesterol alone. When cholesterol and propylthiouracil were combined in the diet, the rise in the blood cholesterol was significantly greater than in the preceding groups. There was a moderate hypercholesterolemia which was approximately six times the average normal values or even ten times the control value in single animals. Increasing the cholesterol content of the propylthiouracil-cholesterol mixture does not appreciably increase the cholesterolemia obtained.

Pathologic Findings.—None of the animals showed any gross atheromatous lesions of the intima of the aorta, of the valves of the heart, or of the major

*It would appear then that the feeding of cholesterol alone in excess of 5 per cent of the diet does not produce any appreciably greater cholesterolemia than that produced by 5 per cent cholesterol or perhaps even lesser amounts of cholesterol in the diet.

arterial trunks. Fatty liver was a common finding in most of the animals which received either cholesterol or thiouracil alone, or the combination. None of the control rats showed evidence of fatty liver.

DISCUSSION

The feeding of diets containing cholesterol in high concentration, alone or combined with propylthiouracil, failed to produce atherosclerosis in the rat. The diets employed resulted in rises in the blood cholesterol levels,* but the levels found did not approach those obtained by Steiner and Kendall[2] in the dog or by ourselves in the chicken.[8] We have been able to demonstrate in the chicken that the development of atherosclerosis is in fairly close relationship to the degree of hypercholesterolemia and its duration.[8] We may assume that the inability to produce a hypercholesterolemia comparable to that seen in Steiner and Kendall's dogs and in our chickens is responsible *in part* for the failure of the rats to develop evidence of atheromatosis. However, it must be noted that cholesterol levels similar to those obtained in the rats in this experiment and maintained for similar periods of time would produce atheromatous changes in both the rabbit and in the chicken.[8, 12] The failure of the rats to develop atheromatosis must be attributed in part to a cholesterol metabolic mechanism different from that which prevails in rabbits and chickens.

Feeding cholesterol in excess of 5 per cent of the total diet did not produce any further rises in the blood cholesterol and this may indicate that there is a threshold for the absorption of cholesterol from the gastrointestinal tract. Cholesterol fed in excess of this threshold concentration is probably excreted unchanged in the feces. Equally tenable hypotheses are: (1) Storage of the additional cholesterol outside the blood stream; (2) endogenous destruction of the additional cholesterol; (3) diminished endogenous synthesis of cholesterol to balance the additional amount ingested; (4) excretion of the additional amount ingested via the bile and the gastrointestinal tract. Only complete cholesterol balance studies can clear up this important question.

Cook and McCullogh[9] fed rats diets containing 2 per cent cholesterol and observed a threefold elevation in the blood cholesterol levels. This is comparable to the elevation we obtained by adding 5 and even 10 per cent of cholesterol to the diet. Cook's balance studies on cholesterol fed rats receiving 2 per cent cholesterol indicated that 30 per cent of the ingested cholesterol could not be accounted for and that the remainder was in the carcass, liver, and feces. He also calculated that the rats absorbed 0.3 to 0.4 Gm. per kilogram per day of cholesterol. If we consider the average daily food intake of our rats receiving 10 per cent cholesterol as 30 Gm., then they ingested 3 Gm. of cholesterol per day. It is probable that nine tenths of this cholesterol passed through the gastrointestinal tract without being absorbed, although proof of this awaits cholesterol balance

*Since no pair fed controls were used in this experiment, we cannot unequivocally rule out the possibility that the relative undernutrition of the thiouracil fed rats may have resulted in cholesterolemia. Other investigators, however, have found that in the rat[13] and dog[14] fasting does not cause any change in the blood cholesterol levels. Likewise, chronic undernutrition in the dog[14] does not cause any significant change in blood cholesterol levels.

studies. It is of interest that the same threshold phenomenon has been observed in the chicken, where cholesterol, fed in excess of 1 per cent of the diet, does not cause any further rise in the blood cholesterol level.[8]

The combination of cholesterol with propylthiouracil produced a synergistic rise in the blood cholesterol levels. The mechanism for this synergism is not well understood. Fleischmann and Shumacker[11] believe that thyroid hormone causes a shift of cholesterol from the blood into the tissues without influencing the total amount of cholesterol in the body. Conversely then, thiouracil by inhibiting the thyroid hormone should arrest this procedure or perhaps reverse its direction, thereby resulting in an increase of cholesterol in the blood.

It has been reported by several observers[9, 10] that cholesterol feeding in the rat results primarily in an increase of the combined cholesterol in the liver with only slight increases in the cholesterol content of other organs. Sperry and Stoyanoff[10] found that in their rats which received a 1 per cent cholesterol diet, as much as 50 per cent of the total body cholesterol was concentrated in the liver. They concluded that omnivores such as the rat did not differ appreciably from the herbivores in their ability to absorb cholesterol and deposit it in the tissues, but that they differed markedly with respect to cholesterol deposition in the arteries.

Thus, while thiouracil fed to the dog diminshed the resistance of that animal to cholesterol-induced atherosclerosis, it failed to do so in the rat. Two possible reasons for this may be considered: (1) We did not use sufficient amounts of propylthiouracil to really disturb the thyroid mechanism (however, in the rat it is difficult to give much greater doses of propylthiouracil than those employed here without causing toxic symptoms to appear), and (2) in the rat, other as yet unknown factors may be involved in this resistance phenomenon which were not affected by the conditions of this experiment.

It would appear then that new avenues of approach must be opened. A combination of endocrine inhibition involving more than one of the endocrine organs concerned with lipid metabolism may shed further light on this problem.

SUMMARY

The feeding of diets containing either cholesterol alone in concentrations of 5 to 10 per cent, or propylthiouracil alone in concentrations of 0.5 to 0.6 per cent, or both combined, for periods up to thirty-eight weeks, failed to produce vascular lesions of the arteriosclerotic variety in rats.

The feeding of cholesterol or propylthiouracil alone produced a cholesterolemia of two to three times the normal values. Combining both gave average values approximately six times normal average values and as much as ten times normal for individual rats.

Cholesterol in excess of 5 per cent did not produce any further rise in the blood cholesterol levels; results of feeding 5 and 10 per cent cholesterol combined with propylthiouracil compared closely with one another.

Proplythiouracil did not succeed in breaking down the rat's resistance to cholesterol-induced atherosclerosis. The resistance to atherosclerosis in this animal is probably dependent on other factors in addition to the thyroid gland and its secretions.

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LABORATORY METHODS

USE OF SPLENIC INFUSION AS A BASE FOR GROWING CERTAIN PATHOGENIC MICROORGANISMS

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NUMEROUS methods for isolating and/or preserving certain microorganisms in their original state for long periods of time have been the object of many investigations.

Lloyd[1] showed that certain accessory growth factors were essential to primary isolation of *Neisseria intracellularis*, and that subsequent to isolation, provided an abundant supply of free amino acid was present, the organisms gradually developed a change in metabolism, becoming increasingly independent of the so-called vitamin content in the medium. The researches of Cole and Lloyd[2] showed that the growth of *Neisseria gonorrhoeae* was considerably dependent upon hydrogen-ion concentration (the mean optimum being pH 7.6), concentration of amino acids, and the presence of certain accessory growth factors or vitamins.

Numerous investigators have reported various media supplying accessory growth factors. Worth,[3] in an effort to provide a universal medium for promoting growth and keeping fastidious microorganisms viable, used a nutrient gelatin containing peptone and sodium chloride. Spray[4] found that certain fastidious organisms grew well upon a hormone-gelatin-agar to which sodium caseinate and soluble starch were added. James[5] found that a 20 per cent infusion of the quahaug, a species of hard-shelled clam, was an excellent source of growth-promoting factor for many of the more fastidious microorganisms.

The lyophile and cryochem methods of Flosdorf and Mudd,[6-8] employing the principle of freezing and rapid dehydration in vacuo, are recognized as superior for the preservation of cultures, but the costs of elaborate equipment and the time and effort expended in maintaining large collections of microorganisms may be prohibitive. The maintenance of bacteria by overlaying the culture with sterile paraffin oil, as recommended by Morton and Pulaski,[9] has advantages but necessitates the use of special selective media for primary growth.

For general and ready use, any culture medium that is simple to prepare, that can be sterilized in the autoclave without subsequent addition of body fluids, and in which the most fastidious microorganisms will grow luxuriantly and remain viable would be ideal. The employment of beef spleen as a growth-promoting factor has fulfilled this need in our own investigations.

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Spleen as a source of growth-promoting factor was suggested by the work of Cole and Lloyd[2] who used a tryptic digest of casein with extract of pig pancreas, which they named tryptamine, and an infusion of beef spleen for growing *N. gonorrhoeae*. It was further suggested by Zinsser and Bayne-Jones[10] who state that Neufeld found that pneumococci remained viable in the desiccated spleens of infected mice for periods far exceeding those on selected media.

To further determine the value of spleen as a factor in growth and viability, the following experiments were conducted.

PROCEDURE

Cultures of eleven of the more fastidious organisms for viability studies were made on various media consisting of splenic infusion agar, splenic infusion gelatin, beef infusion agar, and beef infusion gelatin in varying amounts, with and without the addition of vitamin B_1. Splenic infusion base was prepared in a manner similar to the preparation of beef infusion base, the usual amount of peptone and sodium chloride being added to filtered minced beef spleen infused overnight. The pH was adjusted to 7.6 before autoclaving. Ten per cent gelatin was added to the splenic infusion medium and both 10 and 15 per cent gelatin to the beef infusion medium. When vitamin B_1 was employed in the medium it was sterilized by Seitz filtration and subsequently added aseptically in a 10 per cent concentration.

The organisms used were three strains of streptococci (*Streptococcus pyogenes, Streptococcus viridans, Streptococcus anhemolyticus*), three of pneumococci (*Diplococcus pneumoniae* Types 1, 2, and 3), and one each of *Neisseria intracellularis, Neisseria gonorrhoeae, Hemophilus pertussis, Corynebacterium diphtheriae,* and *Hemophilus influenzae.*

All media were tubed in plastic screw-top vials, 120 by 20 mm., to insure the preservation of moisture during incubation and storage. Prior to inoculation of experimental media, three successive transfers of each organism were made twenty-four hours apart upon selective media for each species.

Inoculations were done with a standard 3 mm. loop from an emulsion of the test organism in mammalian Ringer's solution made to a density corresponding to McFarland nephelometer tube No. 8. This was to insure uniformity of the inoculum of each organism and to prevent a carry-over of nutrient material from the selective medium on which the organisms were growing.

Five tubes of each type of medium were inoculated with the various organisms. After forty-eight hours of incubation at 37° C., growth of each was recorded; the tubes were sealed and placed in the refrigerator at 10° C., with the exception of *N. intracellularis, N. gonorrhoeae,* and *H. influenzae.* The first two were kept in the incubator at 37° C.; the tubes of the latter were immersed in cold water and placed in a dark cupboard at 20° C. Growth in each instance was indicated as negative (–), good (+), and heavy (++). Table I shows the record of growth on various media with appropiate symbols.

With the exceptions of *H. influenzae* and *H. pertussis,* splenic infusion media in every instance, both with and without vitamin B_1, gave rise to excellent initial growth, which was superior to that on beef infusion media.

TABLE I. PROFUSION OF GROWTH OF ORGANISMS AFTER FORTY-EIGHT HOURS OF INCUBATION

ORGANISM	SPLENIC INFUSION BASE				BEEF INFUSION BASE			
	SIA	SIAV	SIG	SIGV	BIAV	BIG10V	BIG15	BIG15V
Str. pyogenes	++	++	+	+	+	+	+	+
Str. viridans	++	++	+	+	++	+	++	++
Str. anhemolyticus	++	++	+	++	++	+	++	++
D. pneumoniae 1	+	+	+	+	+	+	+	+
D. pneumoniae 2	++	++	+	+	++	++	++	++
D. pneumoniae 3	++	++	+	+	+	++	++	++
N. gonorrhoeae	++	++	−	−	+	+	+	+
N. intracellularis	++	++	+	−	+	+	+	+
H. influenzae	−	−	−	−	−	+	+	+
H. pertussis	−	−	−	−	−	−	−	−
C. diphtheriae	++	++	++	++	++	++	++	++

SIA, Splenic infusion agar; SIAV, splenic infusion agar, vitamin B_1; SIG, splenic infusion gelatin; SIGV, splenic infusion gelatin, vitamin B_1; BIAV, beef infusion agar, vitamin B_1; BIG10V, beef infusion gelatin 10 per cent, vitamin B_1; BIG15, beef infusion gelatin 15 per cent; BIG15V, beef infusion gelatin 15 per cent, vitamin B_1.

Transfers from each type of medium were made at intervals of one and one-half, three, six, nine, and twelve months to determine viability. They were made from suspension in Ringer's solution from the growth in the sealed, stored tubes and also by direct transfer to selective media.

Additional studies on splenic medium were made to determine its value as a base for blood agar, its ability to support growth of the pathogenic clostridia, and its use in primary isolation of organisms. For this purpose Petri dish streak cultures of *Str. viridans, D. Pneumoniae, Str. pyogenes, C. diphtheriae,* and *N. intracellularis* were made on plain splenic agar, splenic blood agar, and plain blood agar in order to compare growth and colonial characteristics. Pathogenic clostridia, *Cl. botulinum, Cl. tetani,* and *Cl. novyi,* were grown in deep stabs of splenic infusion agar and broth to determine whether these media would warrant their use for growing anaerobes. Studies on splenic infusion agar were made to evaluate its use in primary isolation of organisms from sputa, nasal washings, and throat cultures. Microorganisms so isolated were grown on splenic infusion agar and vaccines were prepared from these.

RESULTS

Str. pyogenes was found viable on all media except beef infusion gelatin 10 per cent plus vitamin B_1; *Str. viridans* was living only on beef infusion gelatin 15 per cent and beef infusion gelatin 15 per cent plus vitamin B_1; and *Str. anhemolyticus* was living on all the cultural media at the end of one and one-half months. *Str. anhemolyticus* was living on all media and *Str. pyongenes* only on the splenic infusion agar, both with and without vitamin B_1, at the end of twelve months, whereas *Str. viridans* failed to remain alive longer than three months, at which time it was living only on beef infusion gelatin 15 per cent. See Table II.

It will be noted from these results that the strain of *Str. anhemolyticus* employed survived on all types of media for the entire period of the experiment (twelve months) and that *Str. pyogenes* was maintained on splenic infusion agar for a period of one year, but that *Str. viridans* did not survive on any form of splenic media.

TABLE II. SURVIVAL TIME OF ALL ORGANISMS TESTED ON SPLENIC AND ON BEEF INFUSION MEDIA AT VARIOUS INTERVALS

ORGANISMS LIVING AT END OF (MO.)	ORGANISM	SPLENIC INFUSION MEDIA				BEEF INFUSION MEDIA			
		SIA	SIAV	SIG	SIGV	BIAV	BIGV	BIG15	BIG15V
1½	Str. pyogenes	+	+	+	+	+	+	+	+
	Str. viridans	−	−	−	−	−	−	+	+
	Str. anhemolyticus	+	+	+	+	+	+	+	+
	D. pneumoniae 1	+	+	+	+	+	−	+	+
	D. pneumoniae 2	+	+	+	+	+	+	+	+
	D. pneumoniae 3	+	+	+	+	+	+	+	+
	N. gonorrhoeae	+	+	−	−	+	−	+	−
	N. intracellularis	+	+	+	−	+	−	−	−
	H. influenzae	−	−	−	−	−	−	+	−
	H. pertussis	−	−	−	−	−	−	−	−
	C. diphtheriae	+	+	+	+	+	+	+	+
3	Str. pyogenes	+	+	−	+	−	−	+	+
	Str. viridans	−	−	−	−	−	−	+	+
	Str. anhemolyticus	+	+	+	+	+	+	+	+
	D. pneumoniae 1	−	−	+	+	−	−	+	+
	D. pneumoniae 2	−	−	+	+	−	−	+	+
	D. pneumoniae 3	−	−	+	+	−	+	+	+
	C. diphtheriae	+	+	−	+	−	−	+	+
6	Str. pyogenes	+	+	−	−	−	−	+	+
	Str. anhemolyticus	+	+	+	+	+	+	+	+
	D. pneumoniae 1	−	−	+	+	−	−	+	+
	D. pneumoniae 2	−	−	+	+	−	−	+	+
	D. pneumoniae 3	−	−	+	+	−	+	+	+
	C. diphtheriae	+	+	−	−	−	−	−	−
9	Str. pyogenes	+	+	−	−	−	−	+	+
	Str. anhemolyticus	+	+	+	+	+	+	+	+
	D. pneumoniae 3	−	−	−	+	−	−	−	−
	C. diphtheriae	+	+	−	−	−	−	−	−
12	Str. pyogenes	+	+	−	−	−	−	−	−
	Str. anhemolyticus	+	+	+	+	+	+	+	+

The three strains of *D. pneumoniae* survived as follow: At the end of one and one half months, all strains, with the exception of *D. pneumoniae* 1 on beef infusion gelatin 10 per cent plus vitamin B_1, were alive on all media. At the end of six months, survival was recorded on the splenic infusion gelatin, but not on the splenic agar. By the end of nine months all cultures had died.

C. diphtheriae was viable following storage on splenic infusion agar for nine months. On the other hand, similar cultures on any of the beef infusion media failed to survive. *N. intracellularis* and *N. gonorrhoeae* were alive at one and one-half months on splenic agar. Both of these organisms usually require transfers every forty-eight to seventy-two hour on chocolate agar, the medium of choice for their growth. Splenic medium was not conducive to the growth of *H. pertussis* or *H. influenzae*.

Alpha hemolysis of *Str. viridans* and *D. pneumoniae* and beta hemolysis of *Str. pyogenes* were readily distinguishable on splenic blood agar. Colonies of these organisms on splenic blood agar were larger than the usual pinpoint ones on plain blood agar, and the zone of hemolysis was more readily discernible.

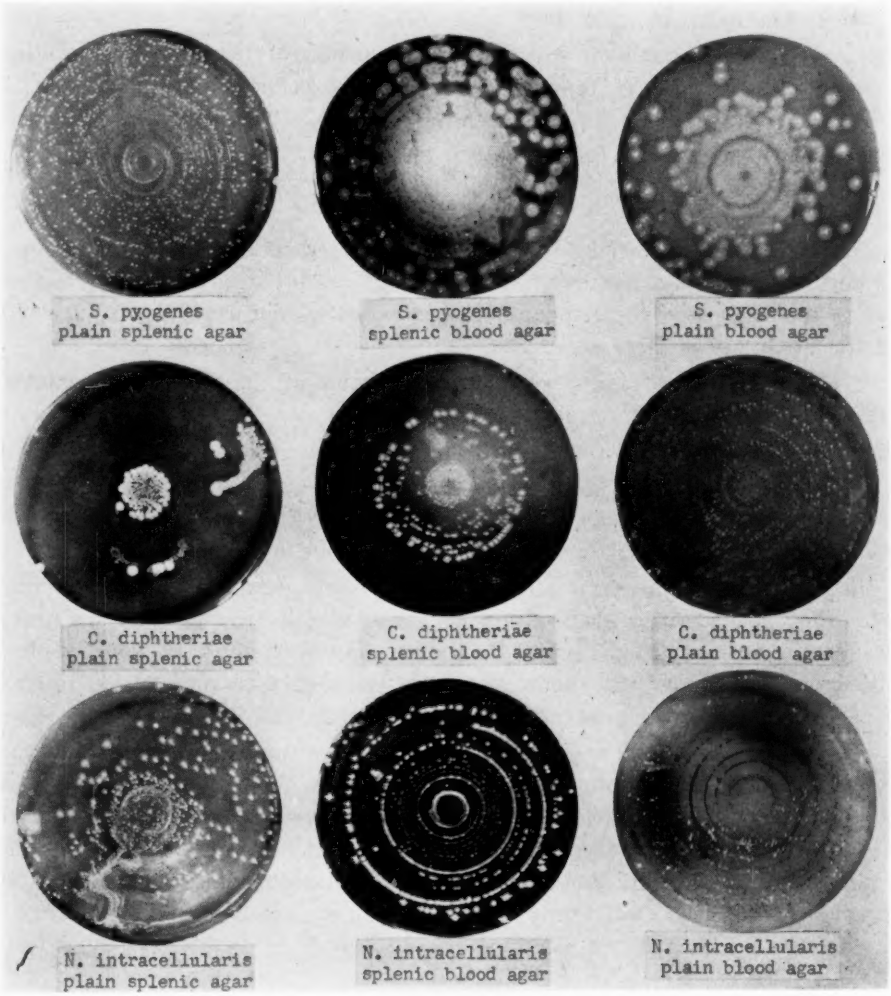

Fig. 1.

Growth and hemolysis were both marked in less than twenty-four hours. Fig. 1 illustrates the excellent growth of these organisms upon plain splenic and splenic blood agar, and the exceptional colony growth and hemolytic action of *Str. pyogenes* on splenic blood agar compared with that upon plain blood agar. The addition of blood to the splenic medium, except for detecting hemolysis, offers no particular enhancement to the growth.

The clostridia were grown in deep stabs of splenic infusion agar and in splenic infusion broth. Growth was excellent and compared favorably with that in ground beef medium or broth to which sterile tissue had been added. Blackening of the agar and broth occurred in deep strata of the medium in a manner similar to the blackening action in brain medium and gelatin as ordinarily observed in certain of the anaerobes.

The profuse growth on splenic infusion media of the organisms studied, except *H. pertussis* and *H. influenzae*, has proved excellent for primary isolation and for the preparation of certain bacterial vaccines. Plain splenic infusion agar furnishes suitable growth for laboratory teaching.

SUMMARY AND CONCLUSIONS

Spleen infusion as a base for agar has been used satisfactorily for maintaining clutures of certain strains of streptococci, *C. diphtheriae*, *N. intracellularis*, and *N. gonorrhoeae* over periods of time in excess of the usual limits for carrying stock cultures of these organisms.

Transfers for carrying stock cultures of most of the fastidious organisms may be made at intervals of one to two months or longer, instead of at weekly or shorter intervals.

A universal medium such as splenic agar is easily prepared, requires no subsequent addition of body fluids, and eliminates the preparation of selective media for growth of each type of the more fastidious organisms to be cultivated.

Luxuriant growth of most of the blood-loving organisms on this medium excels that on a selective medium in each instance. Splenic medium has proved excellent for primary isolation and the preparation of bacterial vaccines. Splenic infusion as a base for broth, agar, or gelatin has been used successfully in classroom work. Pathogenic clostridia grow well in these media.

No studies have been made to determine what growth factor or factors may be present in spleen. Apparently the amino acids in the commerical peptone present, released during bacterial development, plus growth factors from the splenic infusion provide excellent nutrient material. No observable advantage is gained through the addition of vitamin B_1 to splenic media. The preservation of moisture by the use of tightly sealing plastic caps makes possible storage of this medium for several months.

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AN IMPROVED MOUNTING FOR THERMOCOUPLES FOR THE MEASUREMENT OF THE SURFACE TEMPERATURE OF THE BODY

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INTRODUCTION

THE intensity of radiation, measured by a radiometer, provides the best index of the temperature of the outermost surface of the skin. The radiometer is an accurate instrument but is technically difficult to employ; it requires daily calibration; the conversion of electric potentials to temperature is laborious, and an observer or trained subject must hold the instrument in position for each reading. Thermocouples, on the other hand, are technically easy to use, but give inaccurate measurements of surface temperature. This is the result of the type of mounting employed. When a naked thermal junction is used, readings are affected by the temperature of the ambient air, and firm contact between the junction and the skin is difficult to maintain. When the thermocouple is protected from the air by a covering, heat loss from the skin is impeded and the readings are too high. The difference between surface temperature measurements by thermocouple and radiometer is generally 1° to 3° C.[1]

APPARATUS

A thermocouple mounting was developed which allowed the skin surface temperature to be determined with much greater accuracy than previously. This mounting (Fig. 1) was made on a 1 by 3 inch rectangle of copper window screen (16 mesh, wire diameter 0.01 inch). Copper constantan thermocouple wire* was used, and kinking was prevented by plastic spaghetti. Insulation was retained to just beyond the point where the wires passed under the screen, and the insulated portion was lashed to the screen with thread. The naked leads were twisted together and the junction, about one-half inch in length, was soldered to the under side of the screen as indicated. The screen remained quite flexible except for the ends, which were dipped in soft solder to provide firm connections for metal snap buttons. Adjustable bands of elastic cloth were attached to these buttons and held the mounting firmly against the skin.

RESULTS

Surface temperature measurements using these assemblies were compared with readings obtained by radiometer in four different environments. Thermocouples were fastened to the belly, chest, and thighs of nude subjects and temperatures on adjacent skin areas were determined simultaneously by both

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*The most satisfactory wire was nylon-insulated, 7 strand, 36 gauge; obtained from Revere Corporation of America, Wallingford, Conn.

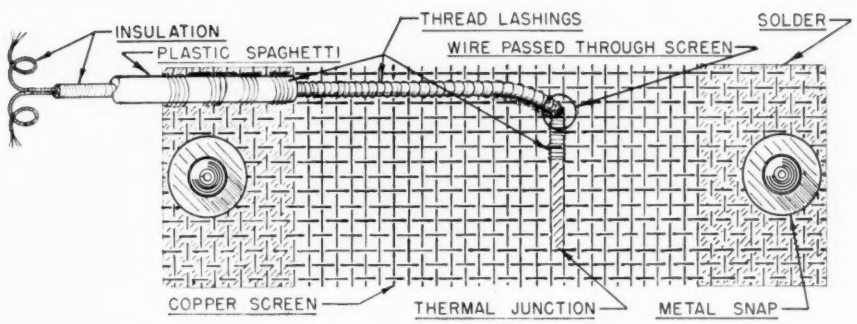

Fig. 1.—The thermocouple assembly.

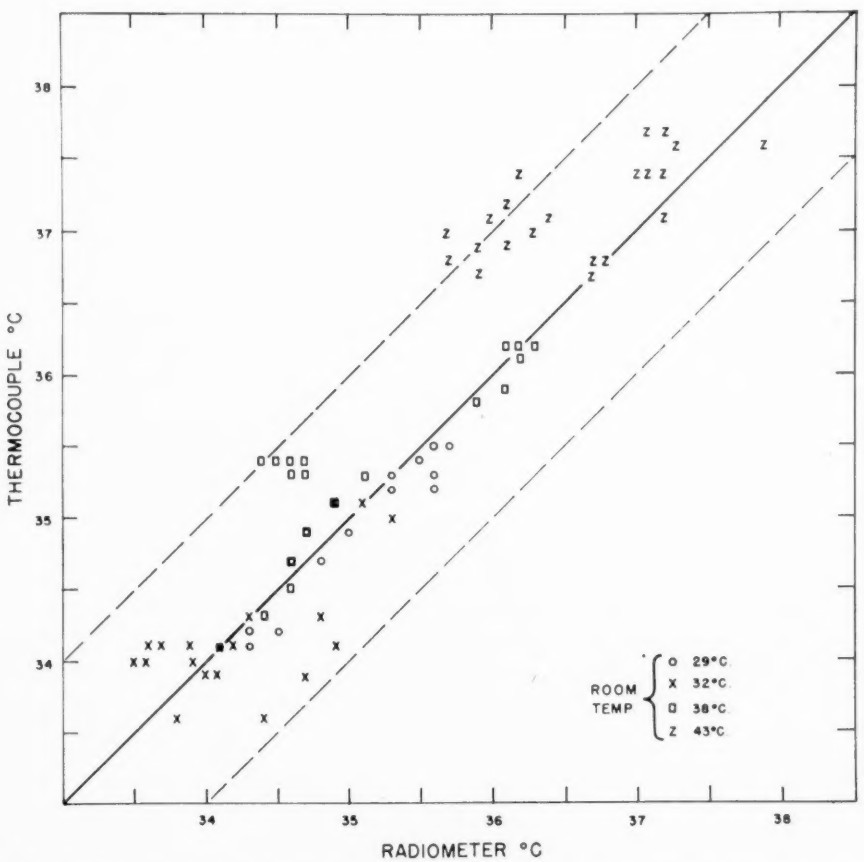

Fig. 2.—Comparison of simultaneous thermocouple and radiometer measurements of surface temperatures.

methods. The results are shown in Fig. 2. The broken lines on each side of the central diagonal show the limits of variations of plus-minus 1° C. between the readings by each method and enclose 95 per cent of all measurements. It was not possible to measure temperature by both methods simultaneously on

exactly the same skin area, and, since the skin temperature may be appreciably different in immediately adjacent areas, a better comparison between the methods is made by comparing the average of a series of readings. Such averages are shown in Table I.

TABLE I. COMPARISON OF THERMOCOUPLE AND RADIOMETER READINGS

ROOM TEMPERATURE (° C.)	NUMBER OF VALUES	AVERAGE SURFACE TEMPERATURE (° C.)		DIFFERENCE (° C.)
		THERMOCOUPLE	RADIOMETER	
29	16	34.9	35.0	−0.1
32	23	34.2	34.2	0.0
38	24	35.4	35.0	+0.4
43	28	37.1	36.5	+0.6

The room temperature still affected the thermocouple readings slightly in the hot environments. The subjects were sweating profusely at these temperatures, and the difference in readings presumably resulted from impaired evaporation. The agreement between radiometer and thermocouple, however, was markedly superior under these circumstances to the agreement when either naked, covered, or partially covered thermocouples with conventional mountings were used. The screen mounting always remained firmly in place despite muscular movement or heavy sweating.

SUMMARY

Thermocouples mounted on copper window screen were designed for the measurement of the skin surface temperatures of human subjects. In environments ranging from 29° to 43° C. the average deviation of a series of thermocouple readings from a simultaneous series of radiometer readings was −0.1° C. in the coolest environment and only +0.6° C. in the hottest. The assemblies were easily constructed; they always remained firmly in place and were quite sturdy.

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STUDIES OF PANCREATIC FUNCTION

IV. A Simplified Method for the Determination of Serum Lipase, Using Aqueous Tributyrin as Substrate, With One Hundred Normal Values by This Method

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IN 1943 Goldstein and Roe[1] published a procedure for estimating serum lipase in which the conditions for the enzyme action were so improved that only one hour's incubation at 37° C. was necessary. In this procedure the substrate was dispersed with bile in the digestion mixture. At that time it was observed that the substrate, tributyrin, could be satisfactorily dispersed in an aqueous medium since tributyrin is slightly soluble in water. Preliminary experiments revealed that if tributyrin is properly homogenized in an aqueous buffered digestion mixture, it is split more rapidly by blood serum than when it is emulsified with bile or bile salts. It seemed desirable to take advantage of this observation since the end point of the titration with the aqueous suspension is more easily discerned and the difficulty of preparing and titrating a bile emulsion is eliminated.

Since previous observations were made with cat serum as the source of the enzyme, it was felt that the experiment should be repeated using the sera of human subjects. We repeated this work using the sera of four students, all apparently in good health. The tributyrinase concentration of each sample of serum was determined in three ways: (1) using the glycerol-bile emulsifying agent as reported in our previous paper, (2) substituting 5 c.c. of distilled water for the glycerol-bile mixture, (3) omitting the glycerol-bile and thus reducing the volume of the digestion mixture by 5 cubic centimeters. The results of this study are recorded in Table I. Our data show that human sera respond in the same way as cat sera to the omission of the glycerol-bile emulsifying reagent. This study also demonstrates that it is not necessary to substitute the distilled water for the glycerol-bile. The determinations were carried out under the conditions outlined in our previous paper.[1]

In order to be sure that this difference in the measurement of tributyrinase activity of human sera represented the limitations of bile and not a difference in the hydrogen-ion concentration of the digestion mixtures caused by the presence or absence of bile, it was necessary to determine the pH of the digestion mixtures before and after hydrolysis. We carried out this experiment using a pH paper method.* The data presented in Table II demonstrate that the differences in hydrolysis are not caused by significant differences in the pH of the digestion mixtures.

From the Department of Biochemistry, School of Medicine, George Washington University.
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*pHydrion paper, Micro-Essential Laboratory, Brooklyn, N. Y.

TABLE I. COMPARISON OF TRIBUTYRINASE ACTIVITY OF HUMAN SERA WITH AND WITHOUT GLYCEROL-BILE

DIGESTION MIXTURE	SERUM			
	1	2	3	4
With glycerol-bile	45	72	37	49
Substitution of distilled water	119	166	115	135
Omission of glycerol-bile	120	172	119	134

Values are expressed as cubic centimeters of 0.1N KOH per 100 c.c. of serum (tributyrinase units).

TABLE II. pH OF THE DIGESTION MIXTURES BEFORE AND AFTER HYDROLYSIS

DIGESTION MIXTURE	BEFORE HYDROLYSIS	AFTER HYDROLYSIS
With glycerol-bile	8.4	8.0
Substitution of distilled water	8.4	8.2
Omission of glycerol-bile	8.4	8.2

The new method developed with the use of an aqueous tributyrin emulsion is described below.

MODIFIED METHOD FOR SERUM TRIBUTYRINASE DETERMINATION

Reagents.—

Substrate: Tributyrin.*

Buffer: Dissolve 5 Gm. of sodium diethylbarbiturate in distilled water and make up to 1 liter.

Calcium Acetate Solution: 20 Gm. of calcium acetate, chemically pure $Ca(CH_3COO)_2.H_2O$, are dissolved in distilled water and the solution is made up to 1 liter.

Alcohol-Ether Inactivating Mixture: To 900 c.c. of 95 per cent ethyl alcohol add 100 c.c. of ether.

Indicator: Dissolve 1 Gm. of phenolphthalein in 100 c.c. of 95 per cent alcohol.

Standard Alkali: A 0.05N solution of potassium hydroxide is prepared.

Procedure.—Pipette 0.75 c.c. of tributyrin, 30 c.c. of the sodium diethylbarbiturate solution, and 30 c.c. of the calcium acetate solution into the receptacle of a hand homogenizer† and pass this mixture through the hand homogenizer three times. Then pipette 20 c.c. portions of the emulsion into two large test tubes, one the control and the other the experimental. Both the control and experimental tubes are placed in a constant temperature water bath at 37° C., and when the emulsions have reached this temperature 1 c.c. of the serum is pipetted into the experimental tube and mixed thoroughly with the emulsion by means of a stirring rod. After one hour of hydrolysis, 1 c.c. of serum is pipetted into the control tube and the enzyme in both tubes is inactivated by pouring each mixture into a 250 c.c. Erlenmeyer flask containing 100 c.c. of the alcohol-ether mixture. The contents of each flask are then titrated with the standard potassium hydroxide solution, using phenolphthalein as the

*Eastman Kodak Company, Rochester, N. Y.
†Schaar and Company, Chicago, Ill.

indicator, to the same shade of light, but definite, pink color. The difference between the two titration values is a measure of the concentration of lipase in the serum. In agreement with conventional methods of designating blood values, we have expressed our results in terms of concentration of enzyme per 100 c.c. of serum. One tributyrinase unit is the amount of enzyme that will catalyze the hydrolysis of tributyrin with the release of 1 c.c. of 0.1N fatty acid in one hour at 37° C. with the reagents and conditions as described.

TABLE III. ELECTROMETRIC TITRATION OF SERUM TRIBUTYRINASE CONCENTRATION (RABBIT SERUM)

SERUM	pH AT POINT OF INACTIVATION	pH AT END POINT OF TITRATION	0.05N KOH (C.C.)	TRIBUTYRINASE UNITS
Control	9.60	10.65	2.51	----
Experimental	9.35	10.65	3.61	55

The final step in this procedure, the titration, can be carried out more accurately as an electrometric titration. We have found the use of a Beckman calomel-glass electrode pH meter (model H-2) in conjunction with an electric stirrer very satisfactory for this titration. In the electrometric titration the end point is at pH 10.65; this corresponds to the end point of the phenolphthalein titration. The difference in the hydrogen-ion concentrations at which phenolphthalein turns pink in an aqueous solution (pH 8.3) as compared with an alcohol-ether medium is due to the suppression of ionization in the alcohol-ether mixture. An example of the electrometric titration for determining serum tributyrinase concentration is presented in Table III.

SERUM TRIBUTYRINASE CONCENTRATION IN ONE HUNDRED NORMAL HUMAN SUBJECTS

Using the technique as outlined, we have determined the tributyrinase concentration of the serum of one hundred persons, including medical students, graduate students, and faculty members. The group consisted of eighty-six men and fourteen women, all apparently in good health. The ages of the group varied roughly from 20 to 50 years, with the majority of the group being in the 20- to 30-year range. All of the samples of blood except seven were drawn in the postabsorptive state; evidence at this time indicates no significant difference in the level of serum tributyrinase before and after meals.

The results of this study of serum tributyrinase levels in one hundred healthy subjects are graphically shown in Fig. 1. The lowest value of this group was 81 tributyrinase units, while the highest concentration was 246 tributyrinase units. There were only three samples of serum with values below 100 tributyrinase units; then there was a sharp rise in the number of samples of serum with values ranging from 100 to 180 tributyrinase units. This was followed by a sharp fall in the number of samples (nine) of serum with values over 180 units. Thus 88 per cent of the samples of serum showed a tributyrinase concentration ranging from 100 to 180 units. Further analysis[2] of these values showed the mean normal to be 146.26, the standard deviation to be 30.05, and the standard error to be 3.01.

Analysis of the values of serum tributyrinase when the samples were divided as to the sex of the donor gives one the impression that the serum tributyrinase of women is decidedly lower than that of men. Fig. 1 portrays this graphically. It is readily observed that the majority of female values fall between 100 and 150 units; the mean normal for the female group was 121.14, the standard deviation 19.11, and the standard error 5.11. When the male group is analyzed it is seen that the majority of values fall between 100 and

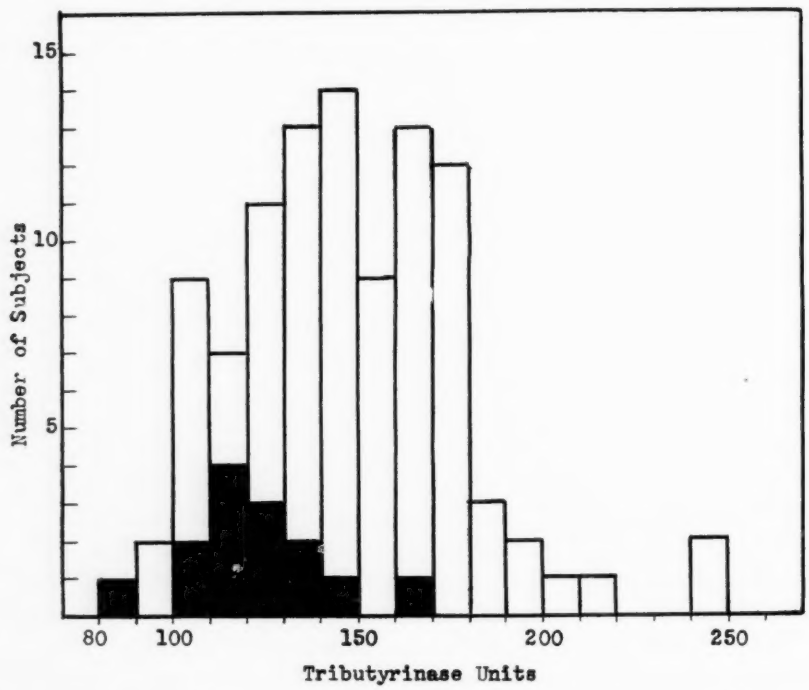

Fig. 1.—Distribution of serum tributyrinase values of one hundred normal human subjects. The height of the column represents the total number of subjects, the area in black, the number of women, and the difference between the two, the number of men.

180 units; the mean normal for the male group was 150.42, the standard deviation 29.45, and the standard error 3.18. In spite of the great discrepancy between the number of samples of serum from men (eighty-six) and those from women (fourteen), the difference between the serum tributyrinase levels of the two sexes is found to be significant when subjected to statistical analysis using the method of the difference between two means ($t = 2.928$; therefore $P < 0.01$).

Utilizing the data further, it can be observed that the 5 per cent fiducial limits for the one hundred determinations of serum tributyrinase will be from 86.55 to 205.97 units. In general it may be inferred that our normal range more roughly will have 85 as the lower limit and 205 as the upper limit. With this range, 97 per cent of the samples of serum have a tributyrinase concentration that can be considered to be within normal limits, 1 per cent is below the lower limit, and 2 per cent are above the upper limit.

SUMMARY

A modification of the serum tributyrinase method, eliminating the use of bile or bile salts, has been developed.

The final step in the procedure, the titration of the fatty acid, may be carried out either with phenolphthalein as the indicator or electrometrically.

The serum tributyrinase level of one hundred normal subjects has been determined.

The range of tributyrinase values for the male subjects was higher than that of the female group.

The normal range of serum tributyrinase levels was found to vary from 85 to 205 units.

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A CLINICAL METHOD FOR THE DETERMINATION OF HUMAN ALBUMIN BY MEANS OF A PRECIPITIN REACTION

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THE need for a rapid and accurate method for the estimation of human albumin in serum or other body fluids in the routine clinical laboratory has long been felt. Among those available methods* which might be adapted to such a purpose are the salt fractionation method of Howe,[1] the methanol precipitation method of Pillemer and Hutchinson,[2] the immunologic method,[3] and electrophoretic analysis with the Tiselius apparatus.[4] Although several modifications[5, 6] have been introduced into Howe's original technique, no procedure which permits a clear-cut separation of albumin and globulins has yet been devised. The chief purpose of our studies is to demonstrate that the precipitin method yields results which approach the accuracy of electrophoretic analysis, the generally accepted standard, and excels the other methods in speed, accuracy, and the saving of labor. Furthermore it permits an accurate determination of albumin present in body fluids in an amount too small to be determined by any other means. The results to be reported in this communication were collected for a period of more than a year from routine analyses in the laboratories of the Squibb Institute for Medical Research and the Sloan-Kettering Institute for Cancer Research.

The principle of the immunologic method is based on the finding that the turbidity produced as a result of the reaction between human albumin and its homologous rabbit antiserum can be used as a measure of the precipitinogen present.

The method for the preparation of the human albumin antigen and the protocol for the immunization of rabbits have been given in a previous paper.[3] A few modifications which have been introduced into the procedure for the albumin determination are described in detail here, and the results of a number of albumin determinations made by the most widely used chemical methods have been included for comparison.

METHODS

Standardization of Pooled Antihuman-Albumin Rabbit Sera.—Each pool of antihuman-albumin rabbit sera was standardized by measuring the turbidity

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The authors are indebted to Miss Lois Hall for technical assistance in the determination of albumin by the immunologic method and to Mrs. Barbara Gottlieb for making the electrophoretic analysis.

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*The method of Milne[6] was published after the collection of our data was started and therefore was not included in this series.

of the immune precipitate produced by the addition of known amounts of human albumin (ranging from 5 to 50 μg of albumin nitrogen) to a sufficient amount of antiserum (2.0 ml.) to insure an excess of antibody. Thus 1 per cent human albumin solution containing 1.474 mg. nitrogen per milliliter by Kjeldahl analysis was prepared by dissolving 1.0 Gm. of electrophoretically homogeneous human albumin in about 50 ml. of 0.85 per cent NaCl solution. This solution was neutralized with enough sodium bicarbonate (approximately 25 mg.) to bring the pH of the solution to 7.6 and then made up to a volume of 100 milliliters. The stock albumin solution was kept in the icebox as the standard; thymol was added as the preservative. In order to standardize a batch of pooled sera, the standard albumin solution was further diluted quantitatively to the following concentrations expressed in micrograms of albumin nitrogen per milliliter: 2.95, 5.90, 7.37, 8.84, 10.32, 11.79, 14.74, 17.69, 20.64, and 29.48. Two milliliters of each of these albumin solutions were added to a series of Klett-Summerson tubes containing 1.0 ml. of 0.85 per cent saline solution. Two milliliters of the rabbit immune serum were then added to all the tubes. The reaction was allowed to proceed at room temperature for at least thirty minutes, and the turbidity of the immune precipitates* was measured at a wave length of 420 millimicrons. A solution containing 2.0 ml. of the antiserum and 3.0 ml. of 0.85 per cent NaCl solution was used as a blank. Under these experimental conditions the antibody in the antiserum was present in such a large excess that the immune precipitate did not flocculate as large aggregates. A uniform suspension with constant and reproducible turbidity readings was obtained by gentle stirring. The turbidity readings, which reached a maximum within thirty minutes, were then plotted against the micrograms of human albumin nitrogen added, Fig. 1.

Determination of Albumin in Human Serum or Plasma.—One-half milliliter of human serum or plasma was diluted to 100 ml. with 0.85 per cent sodium chloride solution, and this solution was used for the determination of the total nitrogen by the micro-Kjeldahl method† and for the determination of albumin concentration. One milliliter of this solution was pipetted into two Klett-Summerson tubes, each containing 2.0 ml. of 0.85 per cent sodium chloride solution for duplicate determinations. Two milliliters of the standardized antiserum were added to each tube. The contents in the tubes were allowed to stand at room temperature for thirty minutes or longer, and the turbidity was measured with a Klett-Summerson photoelectric colorimeter against a control tube containing 2.0 ml. of antiserum and 3.0 ml. of the saline solution. This procedure permits a technician to complete at least fifty albumin determinations in one working day.

*If hemolyzed antihuman-albumin serum was used, the immune precipitate was centrifuged for ten minutes and the supernatant was removed by suction with a fine capillary attached to a vacuum, care being taken not to remove any immune precipitate. Then 5.0 ml. of a normal saline solution were added to suspend the immune precipitates, and the turbidity readings were measured.

†Since we have accumulated our data, it has been found equally accurate to determine the total protein concentration by measuring the turbidity produced by 2.0 ml. of the diluted serum (1:200) and 3.0 ml. of 5 per cent trichloroacetic acid solution. The turbidity of the trichloroacetic acid precipitate bears a quantitative relationship with the total protein nitrogen determined by the Kjeldahl method.

Calculation.—The per cent of the total nitrogen in the sample present as albumin nitrogen (see Table I) was calculated as follows: One can estimate the amount of human albumin (C) corresponding to the turbidity readings (B) from the standard curve (Fig. 1). Multiplying C by the serum dilution (A)

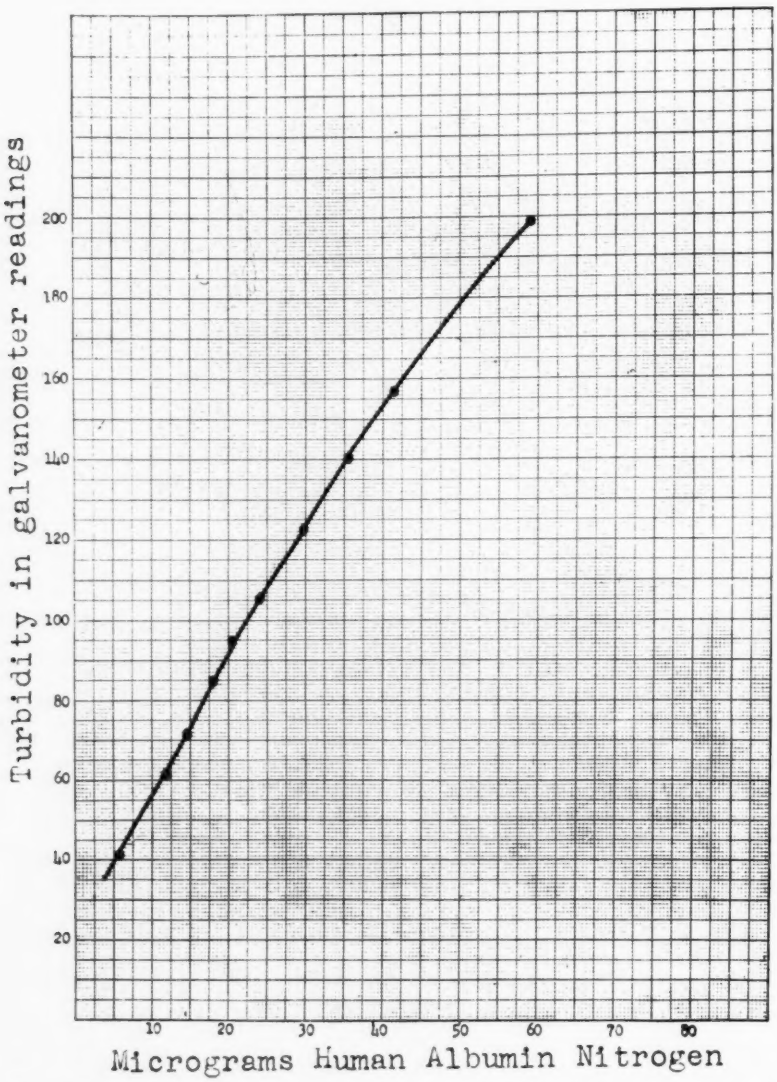

Fig. 1.—The relationship between the turbidity of immune precipitate and the amount of human albumin nitrogen added to 2.0 ml. of its homologous antiserum.

gives the total albumin nitrogen present per milliliter of the original sample (D). The ratio of D to the total nitrogen in milligrams per milliliter of the original serum (E) as determined by the Kjeldahl method or in terms of the turbidity of the trichloroacetic acid precipitate, multiplied by 100, is the per cent

TABLE I. THE CALCULATION OF HUMAN ALBUMIN CONCENTRATION FROM THE TURBIDITY READINGS

SAMPLE	A DILUTION	B TURBIDITY READINGS	C ALBUMIN NITROGEN PER SAMPLE (μG)	D = A × C ALBUMIN NITROGEN PER ML. (MG.)	E TOTAL NITROGEN (MG./ML.)	$F = \frac{D \times 100}{E}$ ALBUMIN (%)
1	1:200	142	36.5	7.30	11.98	61
1	1:400	92	19.5	7.80	11.98	65
2	1:100	139	35.0	3.50	9.93	35
2	1:200	87	18.0	3.60	9.93	36
3	1:100	105	23.5	2.35	12.00	20
3	1:200	59	11.0	2.20	12.00	18

of total nitrogen as albumin nitrogen. The results of the calculation of three samples of sera containing different percentages of albumin are given in Table I for illustration.

RESULTS

The results of the analysis for human sera or plasma albumin by different methods are presented in Table II. The data are arranged according to the total number of analyses, expressed in per cent, which agreed within certain limits with the electrophoretic analyses. They demonstrate that over half (58 per cent) of the samples analyzed by the immunologic method agreed within

TABLE II. RESULTS OF THE DETERMINATION OF HUMAN ALBUMIN BY DIFFERENT METHODS, ARRANGED ACCORDING TO THE DEVIATIONS FROM THE ELECTROPHORETIC ANALYSIS (TAKEN AS 100 PER CENT)

RANGE OF AGREEMENT* (%)	AGREEMENT (%) BETWEEN ELECTROPHORETIC ALBUMIN AND THAT DETERMINED BY:		
	PRECIPITIN REACTION	SALT FRACTIONATION†	METHANOL PRECIPITATION‡
0-5	44	0	15
0-10	58	0	24
0-15	76	0	38
0-20	91	5	47
0-30	98	10	68
0-45	100	64	93
0-55	100	90	100
0-65	100	95	100
0-75	100	100	100
Total number of samples analyzed	144	22	34

*In the calculation of agreement, the figures obtained for albumin by the electrophoretic analysis were assumed to be 100 per cent. These analyses were done in Veronal or Veronal-citrate buffer of ionic strength 0.10 and pH 8.6.

†The Howe method[1] as modified by Robinson and co-workers[5] was used.

‡The precipitation and filtration of the precipitates were performed in an ice bath at about 1° C.

10 per cent, about three-fourths (76 per cent) agreed within 15 per cent, and 91 per cent agreed within 20 per cent of the electrophoretic albumin. On the other hand, none of the results obtained by the salt fractionation method agreed within 15 per cent; 5 per cent of the samples agreed within 20 per cent and only 10 per cent agreed within 30 per cent of the electrophoretic albumin. Similarly, the distribution of the agreement between the electrophoretic and

the alcohol precipitation methods* was as follows: 24 per cent of the analyses was within 10 per cent and 38 per cent was within 15 per cent of the electrophoretic albumin. The data therefore indicate that the routine determination of albumin by the immunologic method approaches the accuracy of the electrophoretic analysis. In the present comparative study the electrophoretic albumin was assumed to be correct and therefore assigned as 100 per cent. This assumption was not totally justified since several errors, as pointed out by Petermann and co-workers,[7] are inherent in the electrophoretic technique. However the cumulative error may amount to only a few per cent.

The analytic results also were grouped according to the per cent of electrophoretic albumin present in the sera. Within each group the agreement between analysis by the electrophoretic and other methods is recorded (see Table III); the figures obtained by the former method were taken as 100 per cent. The data demonstrate that the immunologic results agreed within a few per cent of the electrophoretic albumin for sera ranging from 20 per cent (hypoalbuminemic sera) to 60 per cent (normal sera) of the total serum proteins. In other words, this method is applicable equally to severely hypoalbuminemic sera

TABLE III. COMPARISON OF ANALYTIC RESULTS OF HUMAN ALBUMIN IN SERA CONTAINING DIFFERENT AMOUNTS OF ELECTROPHORETIC ALBUMIN (TAKEN AS 100 PER CENT)

ALBUMIN IN SERA (%)	PER CENT* OF ELECTROPHORETIC ALBUMIN FOUND BY:		
	PRECIPITIN REACTION	SALT FRACTIONATION	METHANOL PRECIPITATION
20-30	101 ± 4.0	195 ± 10	--------
30-40	102 ± 3.6	144 ± 5.2	[illegible]
40-50	105 ± 2.7	142 ± 3.8	121 ± 3.6
50-60	106 ± 3.8	133 ± 1.9	120 ± 4.7

*Standard deviation.

and to normal sera. On the other hand, the agreement between analysis by the electrophoretic and two other chemical methods appears to be best at a high percentage of albumin, and the discrepancy becomes larger as the per cent of albumin in the sera decreases. This situation can be explained on the basis that the so-called albumin obtained according to the fractionation methods contains not only albumin but also alpha globulins.[7] The per cent of alpha globulins in hypoalbuminemic sera is generally much higher than that in normal sera.[8]

Application of the Precipitin Method.—The chief advantages of the immunologic method over either the chemical or electrophoretic methods lie in the small quantity of albumin necessary for each determination and in the specificity of the method. These advantages make it possible to apply this method to the estimation of albumin in fluids of clinical interest. For example, we have followed the disappearance of albumin after an intravenous administration of 75 Gm. of human albumin to a patient with metastatic melanoma. The total circulating plasma protein, as well as the total circulating albumin, was determined shortly before and at various times after injection. The results, given in

*According to our experience, it was essential to use freshly obtained serum samples for the determination with the methanol method; otherwise the results are erratic and may be off as much as 100 per cent or more. The method may be satisfactory in the hands of research chemists but is difficult to control adequately in a clinical laboratory.

Table IV, show that the injection of albumin brought about (1) an increase in total circulating plasma proteins from 168 to 217 Gm., an increase of 49 Gm., and (2) an increase of albumin from 98 to 163 Gm., an increase of 65 grams. The percentage of albumin was raised from 58 to 75 per cent. These effects were demonstrable on the 3¼ hour samples. However, twenty-four hours after injection the total plasma proteins but not the albumin dropped to the preinjection level; thus the per cent of albumin in the total serum proteins remained practically unchanged. When these studies were extended to other types of

TABLE IV. DISAPPEARANCE OF ALBUMIN AFTER AN INTRAVENOUS INJECTION OF 75 GM. OF HUMAN ALBUMIN

TIME AFTER INJECTION (HR.)	T. C. P. (GM.)	T. C. A. (GM.)	ALBUMIN (%)
0	168.1	97.7	58
3-¼	216.8	162.6	75
5	236.7	163.5	69
7-¾	196.8	140.0	71
13-¼	215.0	155.1	72
21-¾	169.9	—	—
24-¼	173.6	123.3	71

T. C. P., Total circulating plasma proteins.
T. C. A., Total circulating albumin.
For the calculation of proteins in circulation, the plasma volume was determined by the method of Gregersen.[11]

cases the rates of disappearance of albumin were found to vary from one patient to another. It is to be emphasized, therefore, that the data presented in Table IV serve only to illustrate the usefulness of the precipitin method and not to demonstrate the rapid disappearance of albumin following albumin transfusion.

Other applications of the immunologic method may include the estimation of the albumin in the urine of nephrotic patients and the determination of albumin in the cerebrospinal fluid. The possible diagnostic significance of the latter determination was pointed out in the preliminary note of Kabat[9] and associates.

DISCUSSION

In this communication we have presented the analytic data obtained by several methods for the determination of human albumin in serum or plasma. Like other investigators, we found that the salt fractionation procedure gave the least reliable results, particularly with plasma from hypoalbuminemic patients, and that the methanol method is an improvement over the salt fractionation procedure. However, Bock[10] concluded from his study that the ammonium sulfate method yielded essentially the same results as the alcohol method. The immunologic method was found to yield results which agreed well with the electrophoretic albumin, regardless of its concentration. Besides being accurate and fast, the immunologic method requires much less material for each test and therefore permits the estimation of albumin in such physiologic fluids as spinal fluid and urine.

In a preliminary note, Kabat[9] and collaborators reported an immunochemical estimation of albumin. Their procedure was very similar to that published by one of us[3] except that the quantity of immune precipitate was measured by Kjeldahl analysis instead of by turbidity. We found that the turbiditiy method

is not only rapid and time saving but also gives results which are sufficiently accurate for a clinical method. It avoids the separation of the immune protein from the other proteins in the sera and the preliminary washings which are required by the Kjeldahl determinations.

SUMMARY

The results of routine estimation of human albumin by different methods were compared. It was found that the immunologic method appeared to be superior to the chemical methods and gave reliable results with sera containing different amounts of albumin. Because of the minute quantity of albumin required for each analysis and the specificity of the test, the immunologic method also has been found useful to estimate the excretion of albumin in the urine of nephrotic patients and to determine the amount of albumin in the spinal fluid.[9]

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